INVESTIGATION OF *MYCOBACTERIUM TUBERCULOSIS* PROTEIN EXPRESSION AND ANALYSIS OF HUMORAL IMMUNE RESPONSES OF TB PATIENTS

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Dissertation presented for approval for the degree of Doctor of Philosophy in Medical Sciences (Medical Biochemistry) at the University of Stellenbosch

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December 2004

Declaration

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work, and has not, previously in its entirety or in part been submitted at any university for a degree.

Signature: Date:

Carmen Pheiffer

Summary

New agents for the diagnosis, prevention and treatment of tuberculosis are urgently required. Yet, despite extensive tuberculosis research over recent years, no new drugs, vaccines or diagnostics have been identified to date. It is widely speculated that the major obstacle to the identification of new therapies is the lack of understanding of the host-pathogen interaction.

This study has investigated whether patterns of antigen expression correlate with molecular epidemiological data and strain virulence through the analysis of protein expression and antigen recognition profiles of different *M. tuberculosis* clinical isolates. Using polyacrylamide gel electrophoresis, enzyme-linked immunosorbent assay, and Western blotting, protein expression and antigen recognition by two genotypically different clinical strains that differed in their frequency in the study population have been compared. In addition to differences in protein expression and antigen recognition between the clinical strains and the reference strain H37Rv, protein expression differences between the clinical strains themselves were observed which may relate to strain frequency and virulence.

Differential protein expression by *M. tuberculosis* strains, may explain the heterogeneous host humoral immune response and why no fully effective serodiagnostic test has been developed to date. To explore this hypothesis, the potential of serodiagnosis in this community, where patients are infected with a wide variety of genotypically distinct strains, was investigated. IgG levels to three mycobacterial antigens showed that serodiagnosis of TB is possible in this community, despite infection by a wide variety of genotypically different *M. tuberculosis* strains. Disease episode affected antibody levels, suggesting that care should be taken when evaluating serological diagnosis for repeat episode patients.

This study has shown that *M. tuberculosis* protein expression is dynamic and that the bacillus presents a hypervariable array of antigens to the host immune system. It is likely that different antigens become immunodominant as antituberculosis chemotherapy progresses, and that these differentially expressed antigens may be tracked as predictors of treatment outcome. This hypothesis was tested by correlating Ag85-specific IgG with treatment response, as assessed by sputum smear conversion after two months of antimycobacterial chemotherapy. No significant correlation between antibody levels and treatment responses was observed, suggesting that antibodies may not be useful surrogate markers or that the incorrect antibody type or mycobacterial antigen were selected. Results were consistent with previous findings where patient-to-patient variation dictated the host humoral response.

The results obtained in this study have demonstrated that although bacteriological factors may influence strain prevalence due to antigen variation and immune evasion, both bacteriological and host factors affect humoral immunity. Differential protein expression by *M. tuberculosis* strains has potentially important implications for serodiagnosis and the development of subunit or DNA vaccines, by suggesting that multi-antigen cocktails should be used. Differential protein expression may also explain why patients do not develop adequate protective immunity and are susceptible to reinfection.

Opsomming

Daar is 'n dringende behoefte vir nuwe middels vir die diagnosering, voorkoming en behandeling van tuberkulose. Ondanks intense tuberkulose navorsing gedurende die afgelope paar jaar, is daar geen nuwe tuberkulose medikasie, vaksines of diagnostiese metodes geïdentifiseer nie. Daar word gespekuleer dat die hoof struikelblok vir die identifisering van nuwe medikasie die onkunde oor die tuberkulose patogeen is.

Deur die analise van proteien-uitdrukking en antigeen-erkenning profiele van verskillende *M. tuberculosis* kliniese isolate is daar tydens hierdie studie ondersoek ingestel of die patroon van antigeen uitdrukking korreleer met molekulêre epidemiologiese data and stam-virulensie. Proteien-uitdrukking en antigeen-erkenning deur twee genotipies verskillende kliniese stamme wat verskil in hul frekwensie in die bestudeerde populasie, is vergelyk deur middel van poli-akrielamied gel elektroforese, ensiem-gekoppelde immuunabsorberende analise en Westelike oordrag. Addisoneel tot die verskille in proteien-uitdrukking en antigeen-ekenning tussen kliniese stamme en die verwysingstam H37Rv, is daar ook verskille aangedui tussen die kliniese stamme self wat kan dui op stam frekwensie en virulensie.

Differensiële proteien-uitdrukking deur *M. tuberculosis* stamme, kan moontlik die heterogene gasheer se humorale immuunreaksie verduidelik en daarmee saam die rede waarom daar nie tot op hede 'n effektiewe sero-diagnostiese toets ontwikkel is nie. Daar is dus ondersoek ingestel na die potensiaal van sero-diagnose in 'n gemeenskap waar pasiënte geïnfekteer is met 'n wye verskeidenheid genotipiese stamme. Die IgG vlakke van drie mikobakteriële antigene het aangedui dat sero-diagnose van tuberkulose moontlik is in hierdie gemeenskap, ten spyte van infektering deur 'n wye verskeidenheid genotipies-verskillende *M. tuberculosis* stamme. Die tussenspel van die siekte het teenliggaampie-vlakke beïnvloed wat

daarop dui dat daar versigtig moet gelet word tydens die evaluering van serologiese diagnose van geïnfekteerde pasiënte wat voorheen siek was.

Hierdie studie toon dat *M. tuberculosis* proteïen-uitdrukking dinamies is en dat die bacillus 'n groot variëteit van antigene tot die immuun sisteem bied. Dit is moontlik dat verskillende antigene immuundominant kan word soos wat antituberkulose chemoterapie toeneem, en dat hierdie verskillend-uitgedrukte antigene as 'n gevolg daarvan gebruik kan word as voorspellers vir behandeling. Hierdie hipotese is getoets deur die korrelering van Ag85-spesifieke IgG met die reaksie op behandeling soos geëvalueer deur speeksel-monster verandering na twee maande se anti-mikobakteriële chemoterapie. Daar was geen noemenswaardige korrelasie tussen teenliggaampie vlakke en die reaksie op behandeling nie, wat daaop dui dat die teenliggaampies nie toepaslike surrogaat merkers is nie of dat die verkeerde teenliggaampie-tipe of mikobakteriële antigeen geselekteer is. Hierdie resultate bevestig vorige bevindinge waar pasiënt-tot-pasiënt verskille die gasheer se humorale immuunreaksie gedikteer het.

Die resultate wat uit hierdie studie volg dui dat alhoewel bakteriologiese faktore die stam-frekwensie kan beïnvloed as gevolg van antigeen-variasie en immuun-ontduiking, kan beide bakteriologiese en gasheer faktore die humorale immuunreaksie beïnvloed. Differensiële proteiën uitdrukking deur 'n verskeidenheid *M. tuberculosis* stamme het potensieël belangrike toepassings vir sero-diagnose en die ontwikkeling van subeenheid of DNS vaksines wat impliseer dat multi-antigeen mengsels gebruik moet word. Differensiële proteiën uitdrukking mag ook verduidelik waarom pasiënte nie 'n voldoende beskermende immuniteit opbou nie en sodoende ontvanklik is vir her-infeksie.

Acknowledgements

I would like to express my sincere gratitude and appreciation to the following people:

My parents… Thank you for your encouragement and support during this study, your belief in me, throughout my academic career, has been my inspiration.

Professor Paul van Helden, my department head and promoter…Thank you for affording me the opportunity to study in your department and for your support and expertise during this study.

Dr. Joanna Betts, my co-promoter… Thank you for your expert guidance and advice during this project.

Dr. Pauline Lukey … Thank you for your helpful contribution to this study.

A special word of thanks to Professor Nulda Beyers and her team for providing me with serum samples from tuberculosis patients, the National Institute of Health and the National Institute of Allergy and Infectious Diseases for monoclonal antibodies made available through the Department of Microbiology at Colorado State University, and Dr. Karin Weldingh of the Statens Serum Institut, Denmark for kindly providing me with HYB76-8.

Friends and Colleagues in the Department of Medical Biochemistry, especially office F409 (Pride, Jeanine and Nico)… Thank you for your assistance, moral support and friendliness during the past few years.

A special word of thanks to Najmeeyah for reading parts of the thesis and to Jeanine for help with Afrikaans translation.

Finally, to my husband Zain… Thank you for your support, patience, and understanding throughout this study.

This study was financially supported by the GlaxoSmithKline Action TB initiative.

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Abbreviations

1D	one-dimensional
2D	two-dimensional
α	alpha
β	beta
δ	delta
γ	gamma
µg	microgram
µl	microlitre
µm	micrometre
/	per
°C	degrees Celsius
A	absorbance
ADC	albumin-dextrose-catalase mycobacterial growth supplement
Ag	antigen
AIDS	acquired immunodeficiency syndrome
ATCC	American type culture collection
BCG	bacille Calmette-Guérin
BCIP/NBT	5-bromo-4-chloro-indolyl-phosphatase/nitroblue tetrazolium
bp	base pair
BSA	bovine serum albumin
CDC	Centre for Disease Control
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CF	culture filtrate
CFP	culture filtrate protein
cm	centimeter
CSF	cerebrospinal fluid
dd	double-distilled
DNA	deoxyribonucleic acid
DOTS	directly observed therapy shortcourse
DTH	delayed type hypersensitivity
DTT	dithiothreitol
ECL	enhanced chemiluminescence
ELISA	enzyme-linked immunosorbent assay
FDA	Food and drug administration
GAS	Glycine Alanine salts
h	hour
HCL	hydrochloric acid
HIV	human immunodeficiency virus
H_2O	water
HPLC	high-performance liquid chromatography
HRP	hydrogen peroxidase
hsp	heat shock protein
H_2SO_4	sulfuric acid
IgG	immunoglobulin G
IL	interleukin
INF	interferon
IPG	immobilised pH gradient
IS*6110*	insertion sequence *6110*

kb	kilobase
kDa	kilodalton
LAM	lipoarabinomannan
LC/MS/MS	liquid chromatography tandem mass spectrometry
LJ	Lowenstein Jensen
log	logarithmic
M	molar
mA	milliampere
mAb	monoclonal antibody
MADCTW	Middlebrooks 7H9 mycobacterial culture medium with 10% ADC enrichment and 0.05% Tween-80
MDR	multiple drug resistance
MGIT	Mycobacteria growth indicator tube
MHC	major histocompatibility complex
min	minute
ml	milliliter
mm	millimeter
MRC	Medical Research Council
MS	mass spectrometry
MWCO	molecular weight cut-off
m/z	mass per charge
NAA	nucleic acid amplification
NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate
NaOH	sodium hydroxide
ng	nanogram
NK	natural killer
nl	nanolitre
NL	Netherlands
nm	nanometer
NRAMP	natural resistance associated macrophage protein
mRNA	messenger ribonucleic acid
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	proline glutamic acid
PPD	purified protein derivative
PPE	proline proline glutamic acid
PMSF	phenylmethylsulfonyl fluoride
PVDF	polyvinylidene fluoride
rBCG	recombinant BCG
RFLP	restriction fragment length polymorphism
rRNA	ribosomal ribonucleic acid
RT	room temperature
SD	standard deviation
SDS	sodium dodecyl sulphate
SLC11A1	solute carrier family 11A member 1
Th	helper T lymphocytes

TB	tuberculosis
TBM	tuberculosis meningitis
TCA	trichloroacetic
TMB	tetramethylbenzidine
TNF	tumour necrosis factor
TOF	time of flight
u	unit
UN	united nations
USA	United States of America
UK	United Kingdom
V	volt
v/v	volume per volume
WCL	whole-cell lysate
WHO	World Health Organization
w/v	weight per volume
ZN	Ziehl-Nielsen

Chapter 1

INTRODUCTION

1.1 Tuberculosis: Burden of Disease

Tuberculosis (TB) is an ancient disease dating back at least to 5000 BC (Sager *et al.* 1972). In 1993 the World Health Organization (WHO) declared TB a global health emergency (WHO, 1993). Despite continuing efforts to control the epidemic, TB is still a major cause of mortality and morbidity worldwide, resulting in approximately 2 million deaths and 8 million new cases per year (Corbett *et al.* 2003). TB is a global problem, although developing countries in Africa, South East Asia and the Western Pacific are the worst hit, accounting for 80% of the world's TB burden (Figure 1.1, 1.2, 1.3).

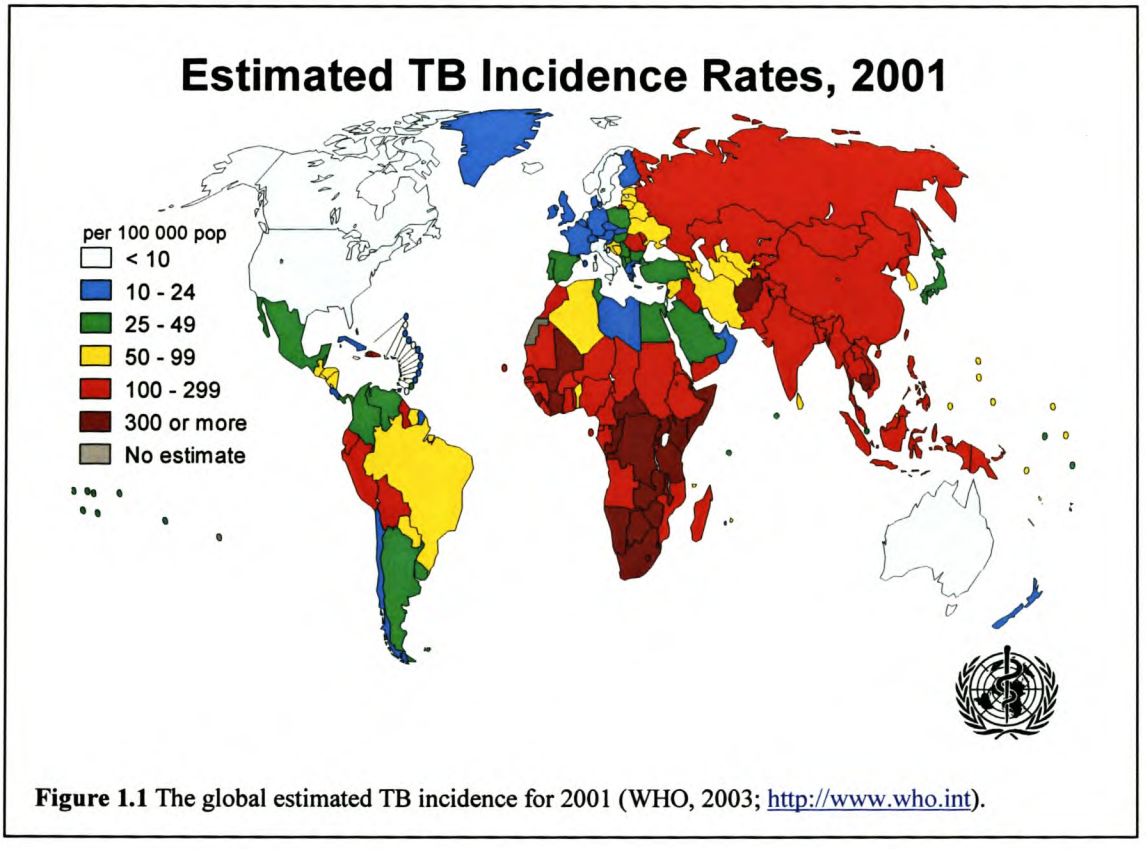

Figure 1.1 The global estimated TB incidence for 2001 (WHO, 2003; http://www.who.int).

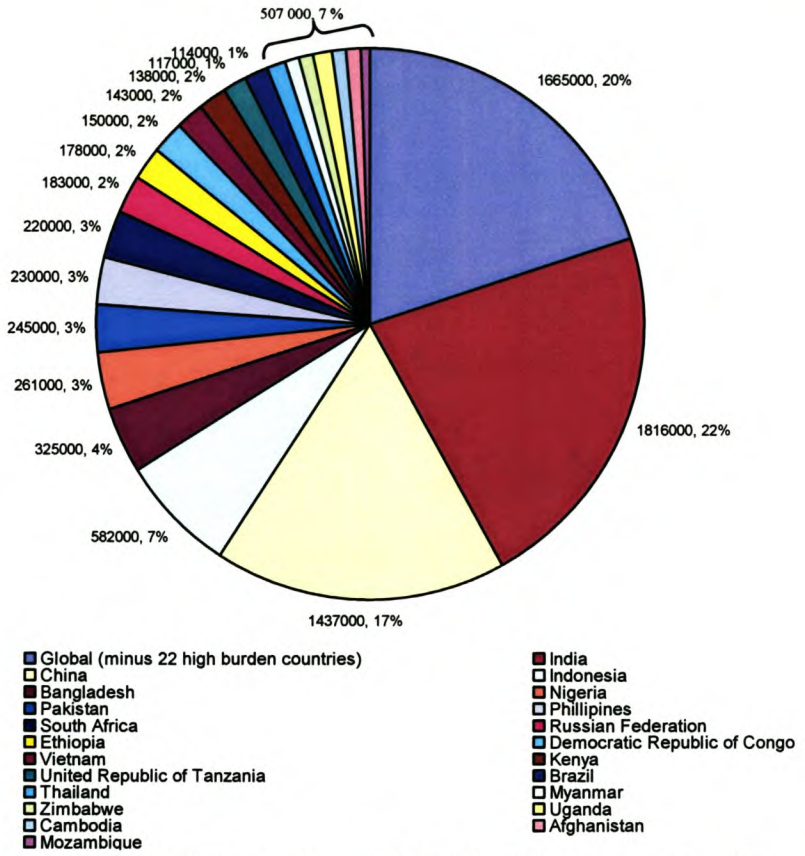

Figure 1.2 The proportion global TB burden in each of the 22 high burden countries. The graph was drawn using estimated TB statistics for 2000 (Corbett *et al.* 2003; WHO, 2003; http://www.who.int).

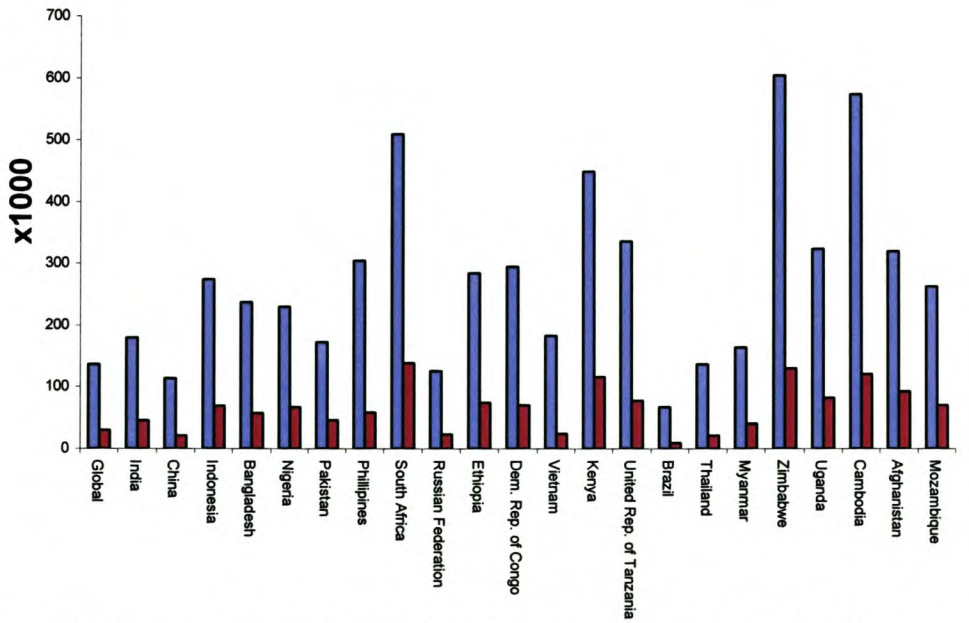

Figure 1.3 The TB incidence (blue) and total number of TB deaths (red) per 100 000, worldwide and for the 22 high burden countries. The graph was drawn using estimated TB statistics for 2000 (Corbett *et al.* 2003; WHO, 2003; http://www.who.int).

1.2 Tuberculosis and Human Immunodeficiency Virus – "A new disease"

Coinfection with TB and human immunodeficiency virus (HIV)/Acquired Immunodeficiency Syndrome (AIDS) is a deadly combination. About 2 billion people (a third of the world's population) are infected with *Mycobacterium tuberculosis*, of whom at least 11 million are coinfected with HIV (Corbett *et al.* 2003; http://www.tballiance.org). TB is the most common cause of death in HIV/AIDS patients, killing one third of these patients worldwide, and up to two thirds in some African countries (http://www.tballiance.org). A linear correlation between HIV seroprevalence and TB incidence in African countries is observed (Figure 1.4). HIV is a major risk factor for reactivation of latent TB, increasing the 10% lifetime risk of reactivation of latent TB to an annual risk of 10% (Bloom and Murray, 1992). HIV/AIDS also accelerates the progression of TB after primary or reinfection (Daley *et al.* 1992; Shafer *et al.* 1995), possibly due to downregulation of cellular immune responses. The effect of HIV/AIDS on TB transmission is unclear, some studies reporting no effect (Migliori *et al.* 1994; Tanzania Tuberculin Survey Collaboration, 2001), while one study has reported that HIV/AIDS does effect TB transmission (Odhiambo *et al.* 1999). TB patients infected with HIV often present with extrapulmonary disease that may be smear negative (De Cock *et al.* 1992) and are considered to be less infectious that HIV negative TB patients (Cruciani *et al.* 2001). Furthermore, TB enhances HIV replication, accelerating the progression to AIDS and death in dually infected patients, and reducing transmission (Goletti *et al* 1996; Toossi *et al.* 2001).

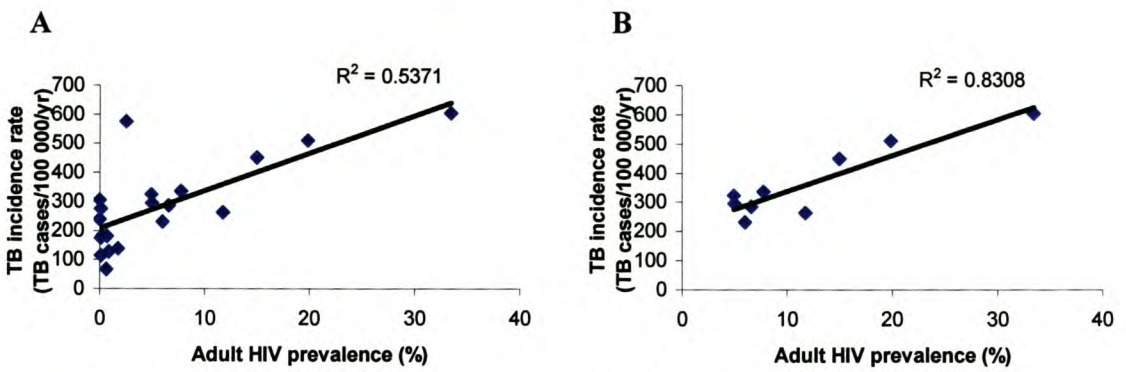

Figure 1.4 HIV prevalence and TB burden. UNAIDS estimates of adult HIV seroprevalence (http://www.unaids.org) and WHO estimates of tuberculosis incidence (Corbett *et al.* 2003) for the 22 high burden countries (A) and for the 9 African high TB burden countries (B).

1.3 *Mycobacterium tuberculosis*

In 1882 Robert Koch identified *M. tuberculosis* as the causative agent of TB (Koch, 1882). The genus *Mycobacterium* consists of more than 70 species that are divided into two groups according to their generation times on solid media (Figure 1.5). Fast growers require less than seven days to form colonies, while slow growers form visible colonies only after seven days (Shinnick and Good, 1994). Slow growers include many pathogenic mycobacterial species, with the tubercle bacillus *M. tuberculosis* being arguably the most important. *M. tuberculosis* is a facultative intracellular pathogen with a cell envelope consisting of peptidoglycan, lipids and glycoplipids such as lipoarabinomannan (LAM). The cell envelope has been implicated in virulence and resistance to certain antibiotics (Daffé and Draper, 1998), and is also responsible for acid-fastness of *M. tuberculosis*, the ability to retain a basic fuchsin dye when treated with an acidified alcohol solution. *M. tuberculosis* has developed successful strategies to evade the host immune response, and is often referred to as the world's most successful pathogen (Hingley-Wilson *et al.* 2003).

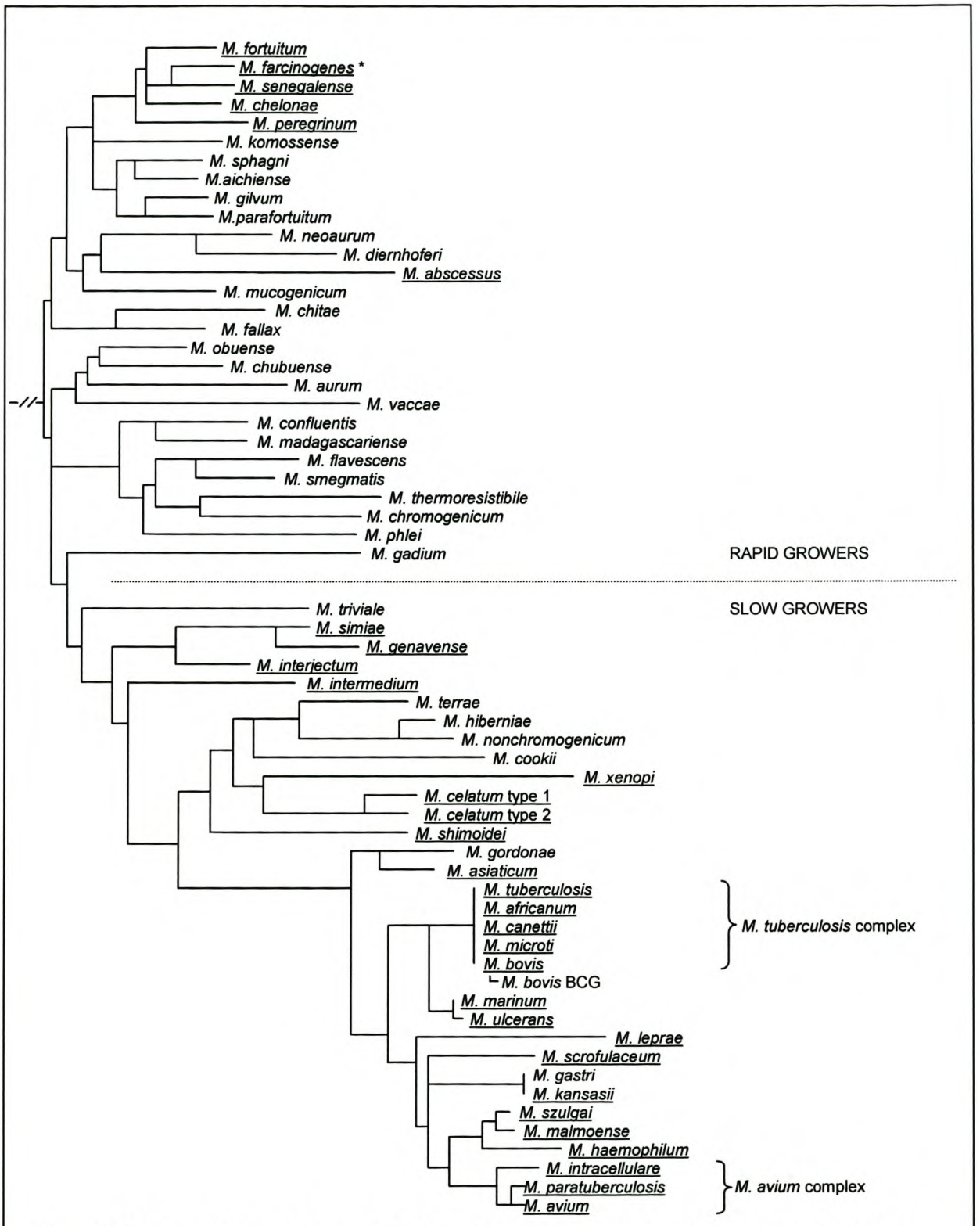

Figure 1.5 Phylogenetic tree of the genus *Mycobacterium*. The phylogenetic tree was constructed using 16S rRNA sequence information. Mycobacteria classified as rapid or slow growers are indicated. **M. farcinogenes* is a slow-growing mycobacterium. Pathogenic species are underlined. Species belonging to the *M. tuberculosis* or *M. avium* complex are indicated. Figure reproduced with kind permission by Dr. NC Gey van Pittius, Department of Medical Biochemistry, University of Stellenbosch, originally adapted from Shinnick and Good, 1994 and Springer *et al.* 1996.

Clinical strains display a wide spectrum of phenotypes (Mitchison *et al.* 1963; Sultan *et al.* 1960). Studies of virulent (*M. tuberculosis* H37Rv) and avirulent (*M. tuberculosis* H37Ra and *Mycobacterium bovis* bacille Calmette-Guérin (BCG)) mycobacteria have shown that this may be mediated by differential capping of LAM, affecting mannose receptor-mediated uptake of mycobacteria and the rate of mycobacterial proliferation within macrophages (Schlesinger *et al.* 1996). Intracellular growth rate (Hoal-van Helden *et al.* 2001a; North and Izzo, 1993a; Silver *et al.* 1998; Zhang *et al.* 1999) and induction of different immunopathological patterns (Bishai *et al.* 1999; Hoal-van Helden *et al.* 2001b; Lopez *et al.* 2003; Manabe *et al.* 2003; Manca *et al.* 1999) may also relate to virulence.

More recently molecular typing of *M. tuberculosis* clinical strains has identified strains thought to be more virulent or prevalent than others. Examples of these include, CDC1551 (Valway *et al.* 1998), the C-strain (Friedman *et al.* 1997), the Beijing or strain W family (Bifani *et al.* 2002; Glynn *et al.* 2002), and subpopulations of the Beijing strain family, strain 210 (Zhang *et al.* 1999) and the W4 strain (Bifani *et al.* 1999). CDC1551 is a clinical strain responsible for widespread infection in a rural community in the United States (Valway *et al.* 1998). It was initially thought that CDC1551 was highly virulent, although later studies have shown that CDC1551 is not more virulent but induces a more rapid and vigorous host immune response (Bishai *et al.* 1999; Manca *et al.* 1999). Friedman *et al.* (1997) reported that the high prevalence of the C strain may be due to intrinsic resistance to reactive nitrogen intermediates produced by activated macrophages. The mechanism for the success of the Beijing strain family, measured by global dissemination, is not known, although Zhang *et al.* (1999) reported that these strains may be able to proliferate better in human macrophages.

Using the rabbit model, Manabe *et al.* (2003) demonstrated that the relative pathogenicity of a specific strain may relate to strain genotype. The genetic variability between clinical isolates has long been exploited by molecular epidemiological studies. Mycobacterial DNA repetitive elements have been most commonly used for genotyping (Chaves *et al.* 1996; Groenen *et al.* 1993; Supply *et al.* 2000; van Embden *et al.* 1993). DNA fingerprinting using the insertion sequence IS*6110* as a probe is the most commonly used method and classifies strains according to the number of IS*6110* elements present within their genome (van Embden *et al.* 1993). Recently genomic deletions and single nucleotide polymorphisms have also been identified as a source of genetic variability (Betts *et al.* 2000; Fleischmann *et al.* 2002).

One of the potentially most interesting sources of genetic variation is between members of the PE (Pro-Glu) and PPE (PPE) protein families. Cole *et al.* (1998) hypothesized that the PE and PPE protein families consisting of 99 and 68 members respectively, may be sources of antigenic variation due to their highly polymorphic C-terminal domains. Variation in the genes encoding these proteins (Betts *et al.* 2000; Fleischmann *et al.* 2002), as well as the mRNA (Flores and Espitia, 2003) and protein (Banu *et al.* 2002) expression patterns between clinical isolates support the hypothesis for their role in antigenic variation. The finding that these proteins are expressed on the cell-surface (Brennan *et al.* 2001; Sampson *et al.* 2002) and are immunodominant antigens (Espitia *et al.* 1999; Choudhary *et al.* 2003; Okkels *et al.* 2003) further support their role in antigenic variation, a possible immune evasion mechanism of *M. tuberculosis*.

1.4 Pathogenesis

The response to *M. tuberculosis* infection, a complex interplay between the pathogen and the host immune system, has a variety of outcomes (Figure 1.6). The immune response of individuals can result in elimination of the pathogen (no infection) or long-term containment, but not elimination of the pathogen in about 90% of individuals (latent infection) (Fenton and Vermeulen, 1996). Individuals with latent infection are identified by a positive tuberculin (PPD) skin test without signs of active disease. About 10% of latently infected individuals may develop active disease during their life (Enarson and Rouillon, 1994). Immunosuppression increases the risk of reactivation of latent infection (Flynn and Chan, 2001), with HIV coinfection being the biggest risk factor for reactivation disease, increasing the lifetime risk of 10% to an annual risk of 10% (Bloom and Murray, 1992).

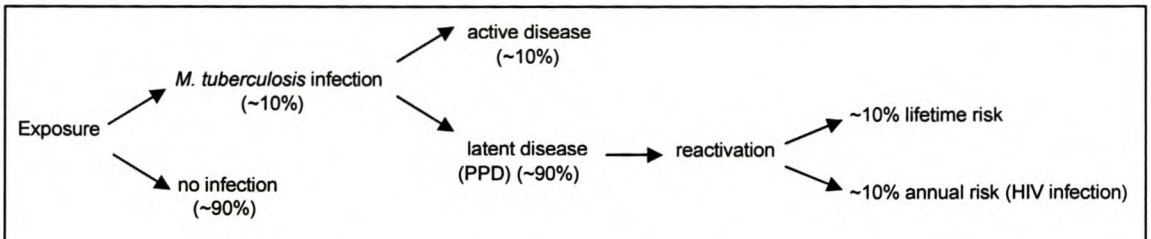

Figure 1.6 Exposure to TB can result in a variety of outcomes.

1.5 Disease pathology

M. tuberculosis can cause disease in any organ, although pulmonary disease is the most common manifestation of tuberculosis in adult humans (Hopewell, 1994). Infection is usually caused by inhalation of aerosol droplets containing *M. tuberculosis* (Riley *et al.* 1959). In the majority of individuals, less than 10% of inhaled *M. tuberculosis* bacilli reach the lung, where

they are phagocytosed by alveoli macrophages (Fenton and Vermeulen, 1996). Macrophages may terminate the infection immediately by destroying bacilli. Alternatively, infected macrophages recruit additional phagocytes, T lymphocytes and natural killer (NK) cells to the site of infection (Flynn and Chan, 2001). Interaction between these cells leads to interferon gamma (IFNγ) production, causing macrophages to become bactericidal (Flesch and Kaufmann, 1987). Activated macrophages secrete tumour necrosis factor alpha (TNFα), which initiates granuloma formation (Flynn *et al.* 1995) (Figure 1.7). The granuloma, a complex network of macrophages and lymphocytes that surround the infected cells (Gonzalez-Juarrero *et al.* 2001; Saunders and Cooper, 2000) creates a hostile environment that restricts mycobacterial growth, resulting in containment of the infection (North and Izzo, 1993b; Saunders *et al.* 1999). Later, granulomas are encapsulated and the centre of the granuloma becomes necrotic. Encapsulation promotes mycobacterial containment and these granulomas are signified by small fibrous and calcified lesions. Alternatively, liquefaction of the granuloma results in the replication and release of mycobacteria from the granuloma, resulting in destruction of lung tissue, and dissemination to other parts of the body (reactivated disease) (Dannenberg and Rook, 1994).

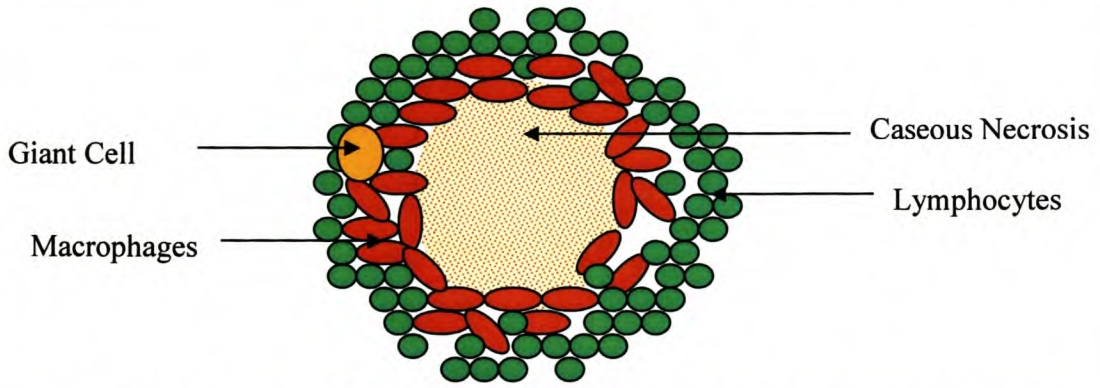

Figure 1.7 Schematic representation of a necrotic granuloma. Figure reproduced with kind permission by Miss L. Moses, PROMEC unit, Medical Research Council, South Africa.

1.6 Diagnosis, treatment and vaccination

DOTS (directly observed therapy shortcourse) is the internationally recommended strategy to control TB, and aims to detect 70% of sputum smear positive cases and attain 85% cure rates by 2005. Since its global introduction in 1995, DOTS coverage increased from 22% to 61% worldwide and from 24% to 61% in high burden countries. By the end of 2001, 155 countries, including all 22 of the highest burden countries had adopted DOTS (WHO, 2003; http://www.who.int) (Figure 1.8).

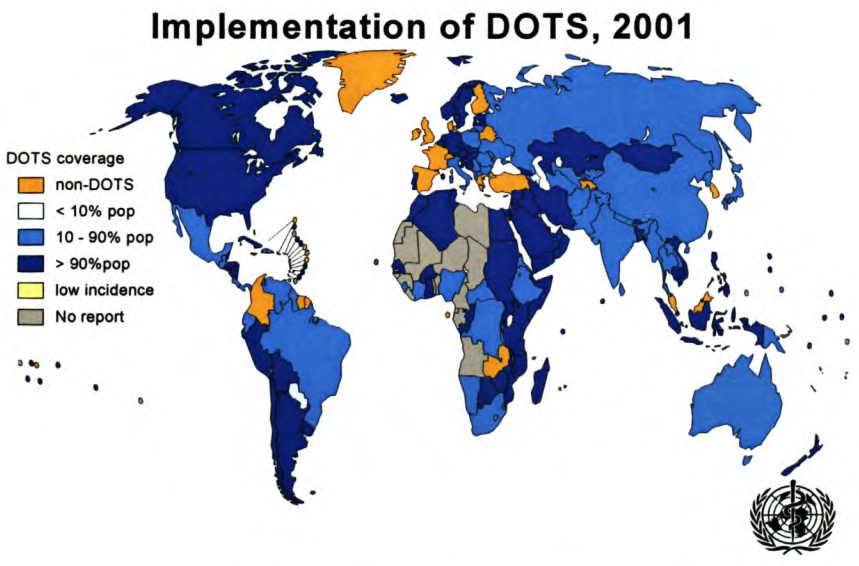

Figure 1.8 Worldwide implementation of DOTS (WHO, 2003; http://www.who.int).

1.6.1 Diagnosis

DOTS detects highly infectious TB cases by medical evaluation (history and risk assessment), chest x-ray or microscopic examination. In 2000, DOTS detected only 27% of the estimated total number of TB cases (WHO, 2002; http://www.who.int), only increasing by 5% by the end of 2001 (WHO, 2003; http://www.who.int). The WHO predicts that if this trend

continues, the 70% target will only be reached by 2012. Explanations for the low case detection rate include: possible over-estimation of the annual incidence of smear positive pulmonary TB, limited or no access to health services, presentation to the private health sector, and incorrect diagnosis due to the quality of the sputum sample, extent of disease, non-specificity, and laboratory error (WHO, 2003; http://www.who.int). Even if the targeted 70%, or best-case scenario, 100% of sputum positive cases are detected, this will not represent all TB. Smear positive TB represents only 45% of the world's total TB burden (Corbett *et al.* 2003). Diagnosis of extrapulmonary and childhood TB is extremely difficult and notoriously unreliable (Bothamley, 1995; Chan *et al.* 2000). The increase in HIV/AIDS further complicates detection of smear positive TB, since coinfected patients often present with extrapulmonary TB and maybe smear negative (Cruciani *et al.* 2001; De Cock *et al.* 1992). DOTS aims to detect infectious cases, however studies have shown that smear negative patients may also transmit TB (Behr *et al.* 1999a; Coleblunders *et al.* 2001).

Clinical symptoms of TB include coughing, fatigue, weight loss, fever and hemoptysis (coughing of blood) (Hopewell, 1994). Risk assessment is by the tuberculin skin test, which identifies exposure. The test involves intradermal injection of PPD, a crude mixture of *M. tuberculosis* extracellular proteins (Seibert and Glenn, 1941), and measurement of delayed type hypersensitivity (DTH) responses (local skin induration and erythma) after 48 to 72 hours (Huebner *et al.* 1993; Snider, 1982). The tuberculin skin test is prone to false-positivity, due to exposure to environmental mycobacteria and BCG vaccination (Daniel and Janicki, 1978; Fine, 1994) and false-negative results, due to anergy, are common in both healthy and immunocompromised individuals (Toossi and Ellner, 1996). New skin tests incorporating *M.*

tuberculosis specific antigens are currently being developed (Colangeli *et al.* 2000; Haga *et al.* 1995).

Clinical suspicion of TB in smear negative patients may be confirmed by bacteriological diagnosis. Bacteriological culture can confirm diagnosis in approximately 60% of smear negative individuals (Levy *et al.* 1989), but due to the slow growth of *M. tuberculosis*, may take up to 6 weeks before a positive culture is obtained. Radiometric methods (BACTEC 460) can confirm smear negative disease after approximately 15 days (Pfyffer *et al.* 1997a, 1997b). New fluorescent-based culture systems (Mycobacteria Growth Indicator Tube (MGIT) and BACTEC 9000 MB) have been developed with promising results (Chew *et al.* 1998; Huang *et al.* 2001; Pfyffer *et al.* 1997a, 1997b). However, these techniques require sophisticated equipment and are therefore currently thought to be too complex and expensive to use in developing countries where they are most needed.

Another avenue of exploration involves nucleic acid amplification (NAA) assays. To date the Food and Drug Administration (FDA) in the USA has approved two NAA tests, the Amplicor *Mycobacterium tuberculosis* test, and the E-MTD test. The Amplicor *Mycobacterium tuberculosis* test involves amplification of the 16S rRNA gene with mycobacterial genus-specific primers. The sensitivity of the test is 79.4-91.9% and 40-73.1% in smear positive and smear negative individuals, respectively (Al Zahrani *et al.* 2000; Bergmann and Woods, 1996; Stauffer *et al.* 1995; Tevere *et al.* 1996). The E-MTD test involves amplification of mycobacterial RNA. This test is able to confirm TB in 90.9-95.2% of smear positive, and in 83-100% of smear negative patients (Bergmann *et al.* 1999; Gamboa *et al.* 1998; Smith *et al.* 1999).

A large number of serological tests with diagnostic potential have been developed, with a number of these being commercially available (Chan *et al.* 2000; Bothamley, 1995; Pottumarthy *et al.* 2000). Evaluation of serodiagnostic tests in populations where they are most needed, namely smear negative, extrapulmonary, tuberculosis meningitis (TBM), and children has demonstrated sensitivities of 70% (Wilkins and Ivanyi, 1990), 73% (Wilkins and Ivanyi, 1990), 82% (Chandramuki *et al.* 2002), and 75.5% (Gupta *et al.* 1997), respectively. Despite these promising results no serological test has been recommended for clinical use. Current research efforts are focused on identifying antibodies to multiple *M. tuberculosis* antigens (Chandramuki *et al.* 2002; Houghton *et al.* 2002), antibodies bound in circulating immune complexes (Raja *et al.* 2002), and antibody-antigen avidity (Arias-Bouda *et al.* 2003).

Another possibility for a diagnostic test is measurement of T cell responses to *M. tuberculosis* species specific antigens. ESAT6, a protein secreted by members of the *M. tuberculosis* complex but not by *M. bovis* BCG (Harboe *et al.* 1996; Behr *et al.* 1999b) and the majority of environmental mycobacteria (Harboe *et al.* 1996; Pollock and Andersen, 1997) is the most promising. Despite doubts being raised about the species specificity of ESAT6 (Gey van Pittius *et al.* 2001), studies have demonstrated the potential of ESAT6 as a diagnostic agent for TB (Doherty *et al.* 2002; Lalvani *et al.* 2001; Lein *et al.* 1999; Ulrichs *et al.* 1998). These studies have shown that ESAT6 is recognized by a variety of different HLA types, identifies subclinical infection, is useful to distinguish between *M. bovis* BCG vaccination and *M. tuberculosis* infection, and can differentiate between pulmonary disease due to *M. tuberculosis* and *M. avium*. Hussain *et al.* (2002) reported that T cell responses to *M. tuberculosis* culture filtrate proteins may also have diagnostic potential.

Diagnostic tests that are dependent on an intact host immune system may be adversely affected by HIV/AIDS, fuelling the development of tests that measure mycobacterial antigen. Measurement of *M. tuberculosis* Ag85 in serum is able to discriminate between TB patients and healthy individuals (Bentley-Hibbert *et al.* 1999; Landowski *et al.* 2001), BCG vaccination and *M. tuberculosis* infection (Landowski *et al.* 2001) and disease due to *M. tuberculosis* and *M. avium* (Bentley-Hibbert *et al.* 1999). Mycobacterial antigen detection in cerebrospinal fluid (CSF) has also shown promise for the diagnosis of TBM (Radhakrishnan *et al.* 1991; Sada *et al.* 1983).

1.6.2 Treatment

TB is treated in a recommended standard protocol usually involving a combination of four drugs, isoniazid, ethambutol or streptomycin, rifampicin and pyrazinamide over a period of six to eight months (http://www.who.int). DOTS treatment is supervised by a health care worker or volunteer, especially during the intensive phase (first two months) of treatment. Patient progress and treatment outcome is monitored, recorded and reported to the national health department. The WHO reported that by the end of 2001, 82% of DOTS registered cases were successfully treated. This varied from 73% in Africa to 92% in the Western Pacific region. The lower level of treatment success in Africa is due to death of 7% of patients, 10% of patients defaulting, 7% of patients being transferred to other clinics without follow up, and 3% of patients were not evaluated. The limitations of the DOTS programme include lack of qualified staff, decentralization of health care systems, lack of political commitment and inadequate drug supply. These have all led to the emergence of multiple drug resistant (MDR) *M. tuberculosis* strains, defined as those strains resistant to at least isoniazid and rifampicin (Kochi *et al.* 1993). At least

4% of TB cases are MDR, and in 1998 the WHO introduced DOTS-Plus, a strategy to aid the management of MDR-TB (http://www.who.int).

No novel TB drugs have been introduced since rifampicin in 1972 (http://www.tballiance.org). Patient default (non-compliance) is linked to the lengthy duration of antimycobacterial chemotherapy, and therefore new drugs, which will shorten the length of treatment, are urgently needed (O'Brien and Nunn, 2001). The biological difficulty of working with *M. tuberculosis*, the lack of investment in tuberculous research by pharmaceutical companies due to the lack of perceived commercial return, the cost of drug development, pressures on pricing and access, and the length and cost of clinical trials are major obstacles facing tuberculosis drug development (Duncan, 2003).

1.6.3 Vaccination

Vaccination is considered the most successful prophylactic measure against infectious disease in general. Most vaccines currently available are targeted against extracellular pathogens and viruses, and induce humoral immunity (Seder and Hill, 2000). However, immunity to TB requires induction of cell-mediated immune responses and is not as simple as influenza, smallpox or polio virus vaccines which only require neutralizing antibodies.

1.6.3.1 The BCG vaccine

The attenuated strain of *Mycobacterium bovis*, Bacille Calmette-Guérin (BCG) was introduced as a vaccine to try to protect against TB in 1921 (Weill-Hallé and Turpin, 1925). Since then it has been administered to more than 3 billion people worldwide, being the most widely used vaccine today (Fine *et al.* 1989). BCG vaccination is considered safe, although

disseminated BCG can occur in individuals who are immunocompromised (Talbot *et al.* 1997) and in those suffering from defects in their IFNγ receptor or IL12 genes (Döffinger *et al.* 2002). BCG confers protection by enhancing cell-mediated immunity and controlling mycobacterial replication after establishment of infection (Marchant *et al.* 1999). BCG is administered to newborns in many countries throughout the world, and may effectively prevent childhood disease. However, the efficacy of BCG against pulmonary disease in adults is questionable (Stern *et al.* 1998). A variety of factors such as BCG strain type, dosage, genetic differences between populations and prior exposure to environmental mycobacteria (Fine, 1995) may explain the variable protective efficacy of BCG. Recently Lopez *et al.* (2003) reported that *M. tuberculosis* strain type may also affect the protection afforded by BCG. Attempts to improve the efficacy of BCG by two successive BCG vaccinations have been unsuccessful (Karonga Trial Prevention Group, 1996). However boosting (Brooks *et al.* 2001) and priming BCG (Feng *et al.* 2001) with vaccines that are not dependent on mycobacterial replication has shown promising results.

1.6.3.2 Vaccine development

The development of a more effective vaccine against TB is a major priority (Andersen, 2001; Brennan and Fruth, 2002). These include the development of both prophylactic and immunotherapeutic vaccines. Areas of vaccine research include the development of recombinant and auxotrophic BCG and *M. tuberculosis*, and subunit vaccines and DNA vaccines expressing antigens of interest.

1.6.3.2.1 Recombinant and auxotrophic vaccines

Live vaccines have the advantage of antigen persistence and inducing optimal protection since they produce most of the antigens present *in vivo* (Andersen, 2001). A recombinant BCG (rBCG) vaccine is BCG modified to express antigens considered to be important, especially those thought to have been lost during the attenuation of BCG (Behr and Small, 1997). Potential rBCG vaccines developed include those expressing mycobacterial antigens (Bao *et al.* 2003; Horwitz *et al.* 2000; Horwitz and Harth, 2003; Pym *et al.* 2003) or Th1 inducing cytokines (Murray *et al.* 1996; O'Donnell *et al.* 1994; Slobbe *et al.* 1999; Young *et al.* 2002).

BCG and *M. tuberculosis* auxotrophic mutants have also been developed. These nutritionally deficient strains are avirulent and may result in active mycobacterial replication, but the duration of the infection in host cells is limited. Chambers *et al.* (2000) reported that a leucine auxotroph of BCG was able to protect guinea pigs, without sensitization to tuberculin, while Guleria *et al.* (1996) developed an auxotrophic mutant that was protective and safe in immunocompromised mice. Auxotrophic mutants of *M. tuberculosis* were shown to be protective in guinea pigs (Hondalus *et al.* 2000; Jackson *et al.* 1999). Deletion of RD1, a region absent from the genome of all BCG strains (Behr *et al.* 1999b), from *M. tuberculosis* resulted in decreased virulence and is a possible candidate for an attenuated vaccine strain (Lewis *et al.* 2003). Studies have also employed signature-tagged mutagenesis to develop attenuated mutants (Camacho *et al.* 1999; Cox *et al.* 1999).

1.6.3.2.2 Subunit and DNA vaccines

Concerns that live vaccines are unsafe in immunocompromised individuals have directed vaccine development toward subunit and DNA vaccines. These vaccines have the added

theoretical advantage of including only protective antigens, while excluding disease-enhancing antigens (Turner *et al.* 2000; Yeremeev *et al.* 2000). Subunit vaccines consist of mycobacterial antigens that have been identified as both immunogenic and protective in a suitable adjuvant. Promising results have been achieved using mycobacterial culture filtrates (Andersen, 1994; Pal and Horwitz, 1992), members of the antigen 85 (Ag85) complex (Horwitz *et al.* 1995), the 38 kDa phosphate-binding protein (Falero-Diaz *et al.* 2000), ESAT6 (Brant *et al.* 2000; Olsen *et al.* 2000), heat shock protein 65 (Silva, 1999) and an Ag85B/ESAT6 fusion protein (Olsen *et al.* 2001). Brandt *et al.* (2002) showed that in contrast to BCG, the efficacy of subunit vaccines is not affected by prior exposure to environmental mycobacteria. Although subunit vaccines and adjuvants are highly specific and have a low risk of side effects, their immunostimulatory and protective efficacy is generally weaker than BCG in animal models.

DNA vaccination involves immunization with a plasmid vector encoding the gene of interest directly into the skin, resulting in endogenous production of the vaccine antigen. DNA vaccination induces both long-lasting humoral and cellular immunity, does not require adjuvants, and is easy and inexpensive to develop (Huygen, 1998). A number of candidate antigens, such as ESAT6 (Kamath *et al.* 1999; Lowrie *et al.* 1999), Ag85 (Kamath *et al.* 1999), Hsp65 (Lowrie *et al.* 1999; Tascon *et al.* 1996), Hsp70 (Lowrie *et al.* 1999), MPT64 (Delogu *et al.* 2000; Kamath *et al.* 1999), MPT63 and MPT83 (Morris *et al.* 2000), and MPT41 (Skeiky *et al.* 2000), have been investigated with variable levels of success. Modifications of conventional DNA vaccination include, "heterologous boosting" or "prime boosting" (McShane *et al.* 2001), co-immunization with multiple DNA vaccines (Kamath *et al.*1999; Morris *et al.* 2000), co-expression of cytokines and mycobacterial proteins (Martin *et al.* 2003), and co-delivery of DNA and subunit vaccines (Kwissa *et al.* 2003).

1.7 Host defence mechanisms

TB immunity involves a complex interplay between macrophages, lymphocytes and cytokines, briefly outlined below.

1.7.1 Macrophages

Macrophages are the first line of defence against invading mycobacteria. They are innate cells that recognize and engulf mycobacteria using a variety of cell surface receptors (Ernst, 1998). After internalization, macrophages process the antigens for presentation to the adaptive immune system. Macrophages may also terminate the infection using a variety of killing mechanisms, such as phagosome acidification and phagosome-lysosome fusion (Deretic and Fratti, 1999) and production of reactive oxygen and nitrogen intermediates (Zahrt and Deretic, 2002).

1.7.2 T lymphocytes

T lymphocytes are involved in protection against TB, and T cells expressing the alpha/beta ($\alpha\beta$) T cell receptor, also known as $CD4^+$ and $CD8^+$ T cells, are crucial for TB immunity. $CD4^+$ T cells are the major effector cells in cell-mediated immunity to TB (Flynn and Chan, 2001). These cells recognize antigens presented by MHC class II molecules on the surface of antigen presenting cells, and secrete cytokines that stimulate intracellular killing of infected macrophages. $CD4^+$ T cells, also known as T helper (Th) cells, are divided into groups Th1 and Th2, according to their pattern of cytokine secretion (Modlin, 1994). $CD8^+$, or cytolytic T cells are also important in protection against TB (Smith and Dockrell, 2000), and recognize antigens presented by MHC class I molecules. These cells lyse infected macrophages, thereby releasing

mycobacteria possibly for ingestion and destruction by more efficient macrophages (Flynn and Chan, 2001). $CD4^+$ T cells are important during active infection, while $CD8^+$ T may be important in protection against reactivation of latent disease (Van Pinxteren *et al.* 2000).

1.7.3 Cytokines

Coordinated expression of cytokines is required for protective immunity. It is thought that a Th1-type response, characterized by production of interleukin 12 (IL12) and IFNγ in particular, is required for protection, while a Th2-type response, associated with production of IL4, amongst others, is considered non-protective. The relative contribution of these cytokine patterns in TB immunity is controversial (Flynn and Chan, 2001; Jung *et al.* 2002). Mycobacterial infection induces IL12 production by macrophages which stimulate the production of IFNγ in activated Th1 cells (Murphy *et al.* 1994). Defects in IL12 (Cooper *et al.* 1997; Frucht and Holland, 1996) and IFNγ (Cooper *et al.* 1993; Flynn *et al.* 1993) production are associated with increased susceptibility to mycobacterial infections. During infection, macrophages secrete TNFα, which activates macrophages to inhibit mycobacterial growth and also promotes granuloma formation (Flynn *et al.* 1995). IFNγ, together with TNFα are the main triggering agents for macrophages to become bactericidal (Flesch and Kaufmann, 1990). Response to TNFα also includes tissue damage over time.

The differentiation of naïve T cells into Th1 or Th2 is determined by the local cytokine environment. IL4 stimulates a Th2 response, while IL12 stimulates a Th1 response (Constant and Bottomly, 1997). A Th2-type response induces B cell proliferation and drives antibody production (Lundgren *et al.* 1989; Punnonen and de Vries, 1994). The dose of antigen affects the

cytokine response, with a very low or very high antigen dose promoting a Th2 response, while a moderate dose predisposes naïve cells to become Th1 (Power *et al.* 1998; Rogers *et al.* 2000). Infection with *M. tuberculosis* induces a Th1 response with a switch to a Th2 response after the infection is cleared, thus preventing host tissue destruction (Sander *et al.* 1995).

1.7.4 Humoral immunity

B cells and antibodies are generally considered non-protective in TB. Studies have reported that cellular and humoral immunity is inversely related (Bhatnager *et al.* 1977; Daniel *et al.* 1981; Lenzini *et al.* 1977), and that antibodies and immune complexes are immunosuppressive in TB (Campa *et al.* 1986; Torres *et al.* 1994). However, recent findings support a role for antibodies in protection against TB. Antibodies to mycobacterial surface-exposed epitopes enhance survival of animals experimentally infected with mycobacteria (Teitelbaum *et al.* 1998), affect clearance and organ distribution of mycobacterial virulence factors (Glatman-Freedman, 2000), interfere with mycobacterial dissemination (Pethe *et al.* 2001), and induce TNFα production (Hussain *et al.* 2000; 2001). A role for B-cells in protection against TB, specifically granuloma formation, was reported by Bosio *et al.* (2000). In 1998 it was proposed that the controversy surrounding the role of antibodies in TB pathogenesis is due to the presence of protective, non-protective, and disease-enhancing antibodies in polyclonal antibody preparations (Glatman-Freedman and Casadevall, 1998).

1.7.5 Host immunogenetics

The importance of host factors in TB immunity is inferred from familial clustering noted as far back as the 17th century (Dubos and Dubos, 1987), and was confirmed by studying disease concordance in monozygotic and dizygotic twins (Comstock *et al.* 1978). More recent studies have reported that MHC affects both $CD4^+$ T cell (Agrewala and Wilkinson, 1999) and antibody responses (Bothamley *et al.* 1989). A number of susceptibility genes have been identified, including the natural resistance associated macrophage protein 1 (NRAMP1) gene, now known as the solute carrier family 11A member 1 (SLC11A1) gene (Bellamy *et al.* 1998a), the vitamin D receptor gene (Bellamy *et al.* 1999) and those encoding proteins essential for the Th1 type immune response (Ottenhoff *et al.* 2002; Rossouw *et al.* 2003).

1.8 Proteomics

Sequencing of the genome of *M. tuberculosis* H37Rv (Cole *et al.* 1998) and CDC1551 (Fleischmann *et al.* 2002), and the development of sensitive mass spectrometry techniques to identify proteins have facilitated the study of *M. tuberculosis* proteomics. The total protein complement of the genome is defined as the "proteome" (Wasinger *et al.* 1995). Analysis of the proteome identifies which genes are really expressed, as the presence of a gene does not imply expression (Andersen *et al.* 1992), and genetic conservation between strains may not mean similar protein expression patterns (Betts *et al.* 2000). A recent study by Jungblut *et al.* (2001) identified 6 proteins not originally predicted from open reading frames (ORFs) from the genome sequence, illustrating the importance of proteomics. Conventional expression proteomics relies on high-resolution two-dimensional (2D) gel electrophoresis, mass spectrometry and database searching for protein identification (Figure 1.9) (Blackstock and Weir, 1999). Using 2D electrophoresis, proteins are separated according to their isoelectric points in the first dimension

(isoelectric focusing (IEF)) and then according to molecular weight in the second dimension (O'Farrell, 1975). Proteins are visualized and quantified by radiolabelling, staining with Coomassie-blue, silver or fluorescent dyes.

Proteomics has been used as a tool to study protein expression under supposed host-relevant conditions in order to gain insight into mechanisms of pathogenicity (Betts *et al.* 2002a; Boon *et al.* 2001; Lee *et al.* 1995; Monahan *et al.* 2001; Yuan *et al.* 1996), identify antigens for subunit or DNA vaccines (Covert *et al.* 2001; Rosenkrands *et al.* 2000a, 2000b; 2002), identify new diagnostic markers (Hendrickson *et al.* 2000), and to identify virulence factors (Betts *et al.* 2000; Jungblut *et al.* 1999; Mattow *et al.* 2001).

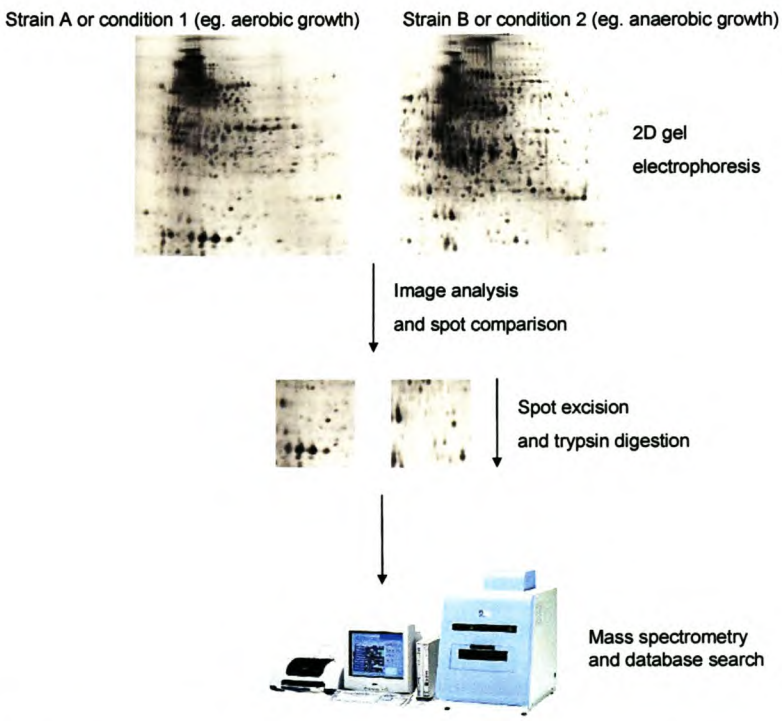

Figure 1.9 Proteomics. Proteins extracted from two different mycobacterial strains or from two different growth conditions are separated by two-dimensional gel electrophoresis. After electrophoresis gels are stained and compared, spots of interest are excised from gels, digested with an enzyme, usually trypsin, and proteins identified using mass spectrometry and database searching (Figure adapted from Betts, 2002b).

1.9 Objectives

1.9.1 Part 1

Protein expression and antigen recognition patterns of clinical isolates will be investigated to gain insight into:

a) Immune evasion mechanisms and strain prevalence

Immune evasion due to differential protein expression has been reported for a number of pathogens (Smith *et al.* 2002). It was decided to investigate this phenomenon in *M. tuberculosis* by comparing protein expression patterns of two local clinical isolates that differ in their frequency in a high incidence TB community (Warren *et al.* 2000). Differential protein expression by these clinical strains may relate to strain prevalence and may explain why strain SAWC1524 is at least 10 fold more frequent than strain SAWC1296 (Warren *et al.* 2000).

b) Exogenous reinfection and failure of serological tests

Differential gene expression by *M. tuberculosis* strains as they infect and cause disease in different individuals may explain why patients are susceptible to exogenous reinfection (van Rie *et al.* 1999a), even after successful cure. Differential gene expression may also explain some of the humoral heterogeneity amongst TB patients (Lyashchenko *et al.* 1998a), and why no serodiagnostic test for TB has been developed to date (Bothamley, 1995; Chan *et al.* 2000).

Protein expression patterns will be analysed using one-dimensional and two-dimensional polyacrylamide gel electrophoresis. Expression of selected *M. tuberculosis* proteins will be monitored by Western blot analysis with monoclonal antibodies specific to the proteins of

interest. Antibodies are markers of antigen expression *in vivo*, therefore antigen presentation by the strains will be analysed using one-and two-dimensional Western blotting, where proteins extracted from each of the three strains will be probed with plasma or sera from TB patients.

1.9.2 Part 2

Investigate antibody responses of TB patients to:

a) assess the potential of serodiagnosis in a high incidence TB setting

Early identification and treatment of TB patients is crucial to limit disease transmission and thereby control the epidemic. However, the current methods used to diagnose TB are inadequate, thereby sparking interest into the development of alternate methods to diagnose TB. Serodiagnosis has shown potential, however, no test has yet been accepted for clinical use (Bothamley, 1995; Chan *et al.* 2000). It is thought that the failure of serodiagnosis is due to the heterogeneous host humoral immune response (Lyashchenko *et al.* 1998a), which is due to, amongst other things, differential gene expression by *M. tuberculosis* strains as they infect and cause disease in different individuals. TB patients in high incidence settings may be infected with a wide variety of genotypically and phenotypically different *M. tuberculosis* strains (van Helden *et al.* 2002). Differential protein expression by these strains may explain the humoral heterogeneity amongst TB patients. To investigate the potential of using serology to diagnose TB in a high incidence setting we will measure IgG levels to three mycobacterial antigens. Furthermore, IgG levels will be correlated with patient smear status, disease episode and the genotype of the infecting *M. tuberculosis* strain.

b) identify a potential biomarker that will predict treatment response

The lengthy course of antimycobacterial chemotherapy results in high levels of treatment failure due to patient non-compliance (WHO, 2003; http://www.who.int). One of the biggest obstacles facing the development of new drugs is the clinical assessment of drug efficacy. The identification of biomarkers, which can be used to speed up the evaluation of new drugs, is urgently needed (Duncan, 2003). Studies have reported that the antibody profile of TB patients changes during antituberculosis chemotherapy (Bothamley *et al.* 1992a; Imaz and Zerbini, 2000). We decided to dissect the IgM and IgG response of TB patients at diagnosis and after four weeks of chemotherapy to determine whether IgM or IgG levels could potentially be used as markers to evaluate treatment efficacy.

This thesis is divided into 8 chapters, consisting of a literature review chapter, six chapters which are presented as manuscripts and a general discussion and conclusion chapter. Introductory material and methodology may be repeated throughout the thesis, because of this. For ease of referencing, literature cited in each of the chapters has been listed at the end.

Chapter 2

OPTIMISATION OF TECHNIQUES

2.1 Introduction

This chapter describes optimisation of some of the techniques used in this study, specifically two-dimensional polyacrylamide gel electrophoresis (chapter 4 and 5), enzyme-linked immunosorbent assay (chapter 3, 4, 5, 6 and 7), and one-dimensional (chapter 3, 4 and 5) and two-dimensional (chapter 4 and 5) Western blotting. The detailed protocols for these techniques are described in the chapters where they are used.

2.2 Two-dimensional polyacrylamide gel electrophoresis

Proteomics is dependent on high resolution two-dimensional (2D) polyacrylamide gel electrophoresis (PAGE), first introduced by O'Farrel in 1975 (O'Farrell, 1975). Proteins, due to their amphoteric nature, are separated by isoelectric focusing in the first dimension, and then separated according to relative molecular weight in the second dimension. Gels are usually stained with silver (detection limit of 1-10 ng) and compared by visual examination or using sophisticated computer programs. If interesting protein spots are noted, gels are rerun and proteins visualized with a stain compatible with mass spectrometry. Spots of interest are excised from the gel, digested with an enzyme, usually trypsin, and proteins identified using mass spectrometry and database searches. The following section describes some of the optimisation steps used.

2.2.1 Sample preparation

M. tuberculosis proteins were extracted by glass bead homogenization in a ribolyser, where the abrasive action of the vortexed beads breaks cell walls, thereby liberating the contents of the cell. Cells were disrupted in a SDS-lysis solution, containing phenylmethylsulfonyl fluoride (PMSF) to inhibit serine proteases. Addition of a cocktail of inhibitors, including serine

protease, cysteine protease and metalloproteases inhibitors (Complete Protease Inhibitor Cocktail, Roche Molecular Biochemicals, Mannheim, Germany) to the lysis buffer resulted in better quality gels (Figure 2.1).

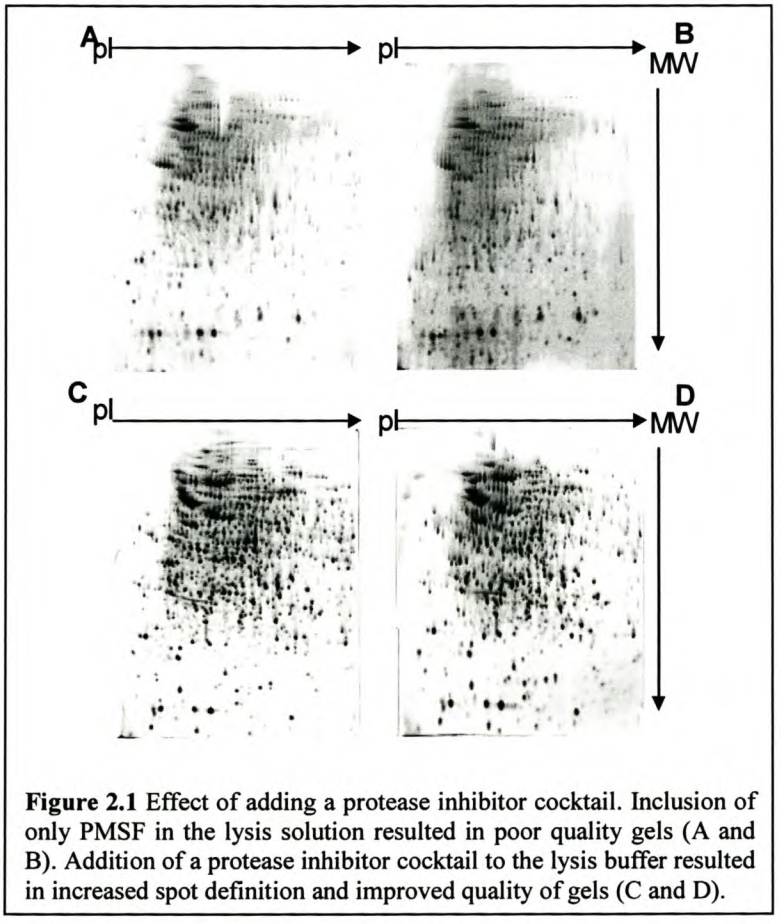

Figure 2.1 Effect of adding a protease inhibitor cocktail. Inclusion of only PMSF in the lysis solution resulted in poor quality gels (A and B). Addition of a protease inhibitor cocktail to the lysis buffer resulted in increased spot definition and improved quality of gels (C and D).

2.2.2 Isoelectric focusing

Proteins were separated in the first dimension using commercially available immobilised pH gradient (IPG) strips (Görg *et al.* 2000). Due to ease and high protein loading ability, rehydration of IPG strips in the presence of sample is preferred to sample cup application. These two methods of sample loading, using two different concentrations of sample and two different running conditions, were compared. Results showed no significant differences between the two

methods, although the use of sample cups resulted in some precipitation at the point of application (Figure 2.2). Rehydration loading was adopted for all future work. Neither isoelectric focusing using 43 600 Vh or 61 100 Vh, nor using 15 µg or 20 µg of protein significantly affected protein separation (Figure 2.2). Improved sample separation was obtained using pH 4-7 IPG strips compared to pH 3-10 IPG strips (data not shown). Horizontal streaking or incompletely focused spots, possibly due to ionic impurities in the sample, was observed. Instead of desalting the sample by dialysis, or precipitation with trichloroacetic (TCA) acid and acetone, which can result in protein losses, the isoelectric focusing protocol was modified, by adding a pre-step of 100 V for 2 hours, allowing the ions in the sample to move to the ends of the IPG strip and resulting in better quality gels (data not shown).

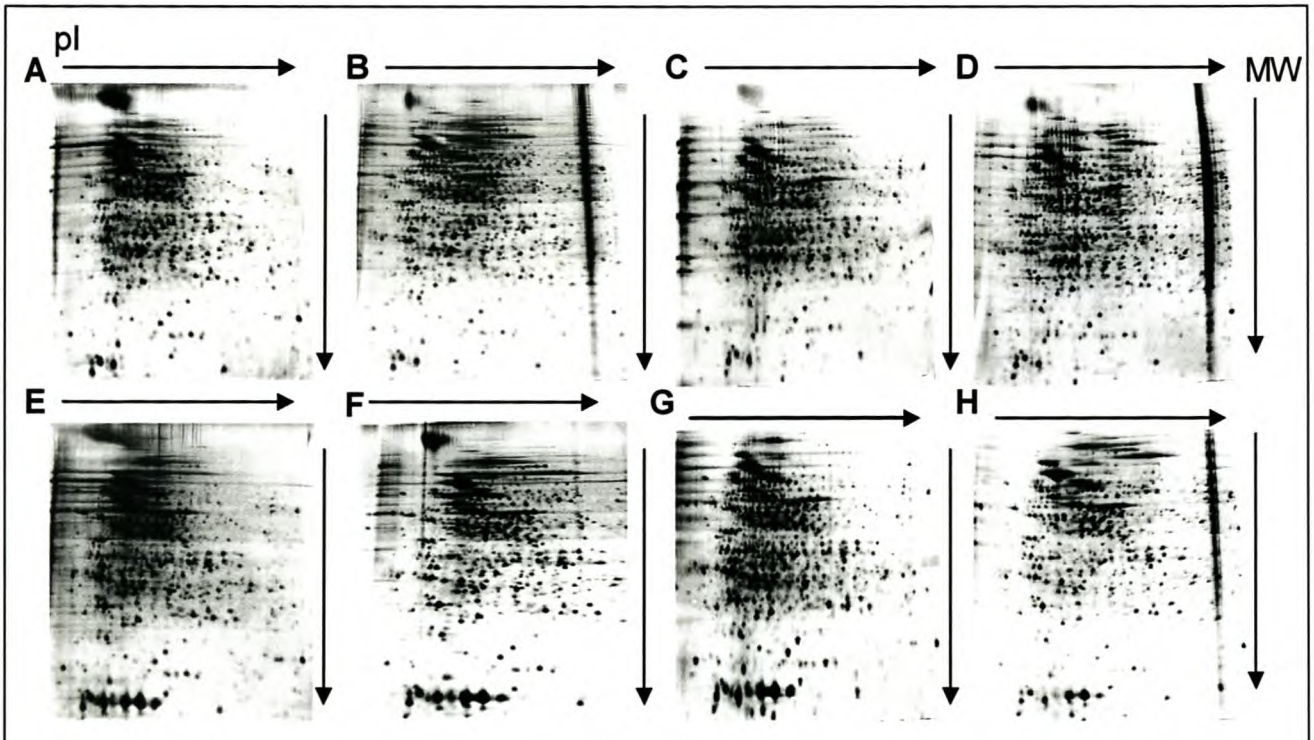

Figure 2.2 Isoelectric focusing conditions. Addition of samples to rehydration buffer (A, C, E, G) and to sample cups (B, D, F, H) were compared using 20 µg of protein and 43 600 Vh (A, B, E, F) and 15 µg of protein and 61 100 Vh (C, D, G, H). Arrows indicate the direction in which the first and second dimension electrophoresis was performed. The vertical smears on the gels represent sample precipitation due to sample cup loading.

2.3 Enzyme-linked immunosorbent assay

The quantification of antigens and antibodies in biological samples has been facilitated by the enzyme-linked immunosorbent assay (ELISA), which was first described in 1971 (Engvall and Perlman, 1971). Subsequent adaptation of the ELISA to the microtiter plate format has resulted in widespread application (Daniel and Debanne, 1987). Antibodies, antigens or carbohydrates are passively adsorbed to a solid-phase, usually plastic, whereafter subsequent reagents are added and unreacted materials are washed away. The addition of an enzyme-labelled reagent, followed by the relevant substrate, causes a colour change from which the biological activity of the sample can be determined. In this study, indirect and sandwich ELISAs were applied to quantify antigen in a complex mixture of *M. tuberculosis* proteins using monoclonal antibodies (mAbs) specific to the antigen of interest, and to quantify antibody (IgM, IgG and IgG subclasses) responses in TB patients. This section will discuss some of the steps used to optimise the indirect ELISA. Optimisation of the sandwich ELISA will be discussed in chapter 7.

2.3.1 Antigen concentration

It is important to assess the effect of different antigen concentrations, since antigen concentration may affect the outcome of the ELISA. To determine the optimal concentration of whole-cell lysate and culture filtrate proteins for coating we used Purified Protein Derivative (PPD), since PPD was readily available and also consists of a complex mixture of *M. tuberculosis* proteins. The optimal concentration of PPD was determined by titrating various concentrations against plasma from TB and control individuals (Figure 2.3). A plateau region was observed when coating wells of ELISA plates with 0.5 μg of PPD, implying that the binding capacity of the polystyrene plates has been reached and that no more antigen can bind. High

concentrations of antigen may inhibit antibody binding through steric hindrance, or may increase stacking or layering of antigen, resulting in a less stable interaction with subsequent reagents (Cantarero *et al.* 1980).

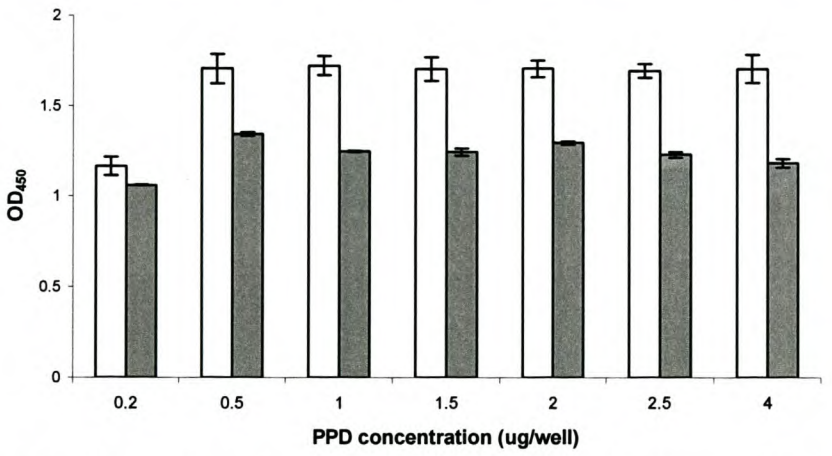

Figure 2.3 ELISA to optimise antigen concentration. Concentrations of PPD ranging from 0.2 µg to 4 µg per well were probed with plasma from TB (clear bars) and control individuals (shaded bars). Each bar represents the average of two measurements. Error bars show the standard deviations from the mean.

2.3.2 Secondary antibody concentration

The optimal dilution of horseradish peroxidase (HRP) conjugated goat anti-human immunoglobulin G (IgG) was determined by titrating various dilutions (1:1 000; 1:2 500; 1:5 000; 1:10 000; 1:12 500; 1:15 000) against PPD. Dilutions between 1:1 000 and 1:12 500 resulted in no significant difference (Figure 2.4), suggesting that saturating concentrations were achieved. A 1:10 000 dilution was used for future work because it occurred in a non-critical region where the OD values plateau.

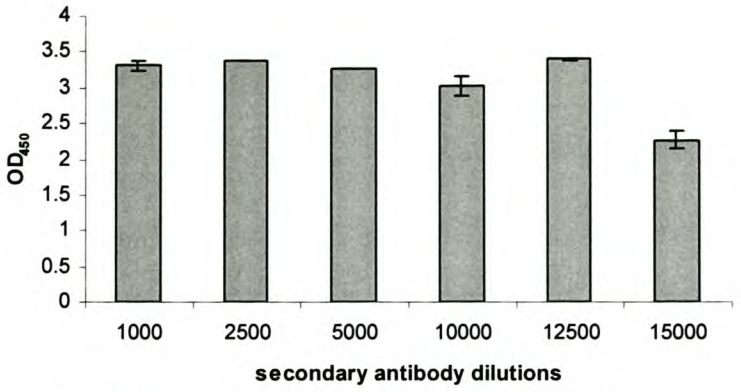

Figure 2.4 ELISA to optimise secondary antibody concentration. The optimal dilution of HRP-conjugated goat anti-human IgG was determined by titrating various dilutions against 1 μg per well of PPD, using a 1:100 dilution of plasma from a TB patient. Each bar represents the average of two measurements. Error bars show the standard deviations from the mean.

2.3.3 Plasma dilution

The effect of plasma dilution on reactivity of samples from TB and control individuals was investigated. Various dilutions of plasma were tested against PPD (Figure 2.5). A stepwise decrease in reactivity was observed using higher dilutions of plasma, with a dilution of 1:10 showing the highest reactivity, and a dilution of 1:400 showing the lowest reactivity for both TB and control individuals. Results suggest that a number of plasma dilutions should be analysed since single dilutions may be inaccurate, for example a 1:400 plasma dilution did not accurately distinguish between TB patients and controls in this investigation.

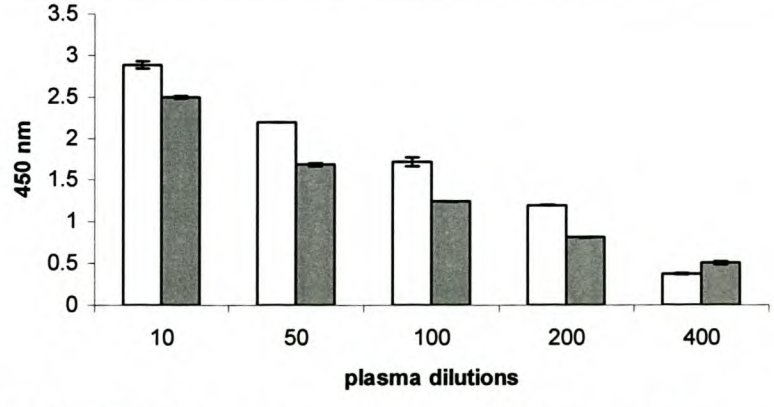

Figure 2.5 The effect of plasma dilution on reactivity of samples. Plasma samples from TB (clear bars) and control (shaded bars) individuals were diluted between 1:10 and 1:400 and reacted with PPD. Each bar represents the average of two measurements. Error bars show the standard deviations from the mean.

2.3.4 Discrimination between TB patients and control individuals

The use of serodiagnosis for tuberculosis is hampered by the presence of cross-reactive antibodies in control individuals (Laal *et al.* 1997). It was hypothesized that optimal ELISA conditions should allow better discrimination between TB patients and control individuals. To determine the effect of plasma dilution and incubation temperature on the discrimination between TB and control individuals, different dilutions of plasma at room temperature (RT) and at 37°C were analysed. Plasma samples were probed against PPD, *M. tuberculosis* H37Rv whole-cell lysate and culture filtrate proteins (CFP), and *E. coli* whole-cell lysate proteins. Table 2.1 shows the ratios obtained by dividing the OD_{450} readings obtained with TB plasma by the OD_{450} readings obtained with control individuals, for each of the antigens tested at RT and at 37°C. The best discrimination between TB patients and control individuals (highest ratio) was achieved using 1:300 plasma dilutions, possibly due to the diluting out of non-specificity. The discrimination between patients and controls was further improved by incubation at 37°C. The difference between TB and control individuals was greater (higher ratios) when measuring reactivity against *M. tuberculosis* antigens compared to *E. coli* antigens (smaller ratios). This is expected because all individuals (healthy or diseased) have *E. coli* reactive antibodies (Laal *et al.* 1997). For *M. tuberculosis* antigens, the discrimination between TB patients and control individuals was greater for PPD and *M. tuberculosis* culture filtrate proteins compared to whole-cell lysate proteins. Whole-cell lysates contain most of the regulatory proteins which are conserved throughout bacteria (Shinnick *et al.* 1988), whereas culture filtrates contain mostly secreted proteins, which are specific to *M. tuberculosis* (Andersen *et al.* 2000).

Table 2.1 Response of TB and control individuals towards PPD, *M. tuberculosis* H37Rv whole-cell lysate (WCL) and culture filtrate (CF) proteins, and *E. coli* whole-cell lysate (WCL) proteins. The differences between the two groups of individuals were calculated according to the formula $^{450\text{ nm TB}}/_{450\text{ nm control}}$, so that the numbers represent the ratio between TB and control individuals as explained.

	RT			37°C		
	1:200	1:300	1:400	1:200	1:300	1:400
PPD	5.62	7.66	6.6	6.44	8.34	7.62
H37Rv WCL	3.22	4.89	3.6	3.9	5.9	6.2
H37Rv CF	5.28	7.54	6.77	5.46	8	7.6
***E.coli* WCL**	1.4	1.48	1.2	1.33	1.61	1.29

2.4 Western blotting

The combination of gel electrophoresis and specific localization of antigen on the basis of its interaction with a specific antibody and its relative molecular weight was first described by Burridge in 1976 (Burridge, 1976). In his approach, antigen was directly detected on the gel, although transfer of proteins from the gel onto a membrane support, usually nitrocellulose, and then probing with specific antibodies has now become the method of choice (Burnette, 1981; Towbin *et al.* 1979). Western blotting is a robust, relatively simple, and sensitive technique and is a commonly used technique to calculate the relative abundance of a specific protein in a complex mixture, or to characterize new antibody preparations. Most gel electrophoresis techniques involve denaturation of the protein sample, so the major constraint of Western blotting is that antibodies will only bind denaturation-resistant (linear) epitopes. In this study, Western blot analysis was applied to analyse expression of specific *M. tuberculosis* proteins by probing crude cell lysates and culture filtrates with mAbs, and to investigate the host humoral immune response to *M. tuberculosis* antigens. For increased resolution, both 1D and 2D Western blotting were

used. Western blotting using mAbs did not require optimisation as they were diluted according to the manufacturer's instructions. Western blotting with patient samples required optimisation, and will be discussed.

2.4.1 One-dimensional Western blotting

Western blot analysis using minigels showed that TB patients and control individuals generally recognised the same proteins in whole-cell lysates and culture filtrates from *M. tuberculosis* H37Rv and *M. bovis* BCG (Figure 2.6). Quantitative differences between TB and control individuals were noted, which has been previously reported by Rojas-Espinosa *et al.* (1999). To improve resolution so that differences between TB and control individuals could be identified, Western blotting with larger format gels (14x14 cm) was attempted (Figure 2.7). Larger gels resulted in improved resolution, although a broad diffuse smear between 30 kDa and 40 kDa obscured proteins in this region. This smear probably corresponds to lipoarabinomannan (LAM) (Hunter *et al.* 1986), a cell wall glycolipid and virulence factor (Daffé and Draper, 1998). High backgrounds, which varied from sample to sample, were observed (data not shown). This is possibly due to the sensitivity of the detection system used, prompting us to investigate alternate detection systems.

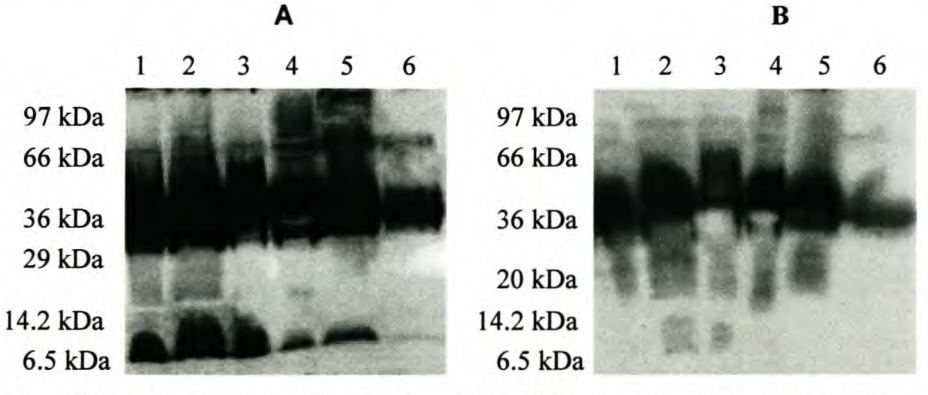

Figure 2.6 Western blot analysis of TB and control individuals using minigels. Ten micrograms of *M. tuberculosis* H37Rv whole-cell lysate (lanes 1, 2), *M. bovis* BCG whole-cell lysate (lane 3), H37Rv culture filtrate (lanes 4, 5), and BCG culture filtrate (lane 6) was separated by 12% SDS-PAGE, transferred to nitrocellulose, and probed with plasma from TB patients (A) and control individuals (B).

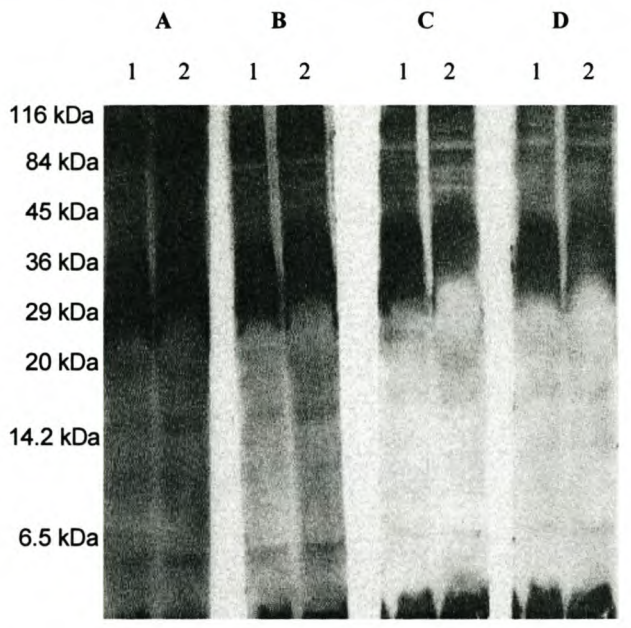

Figure 2.7 Western blot analysis of TB and control individuals using large gels. *M. tuberculosis* H37Rv culture filtrate proteins (50 µg, lane 1; 100 µg lane 2) was separated by 12% SDS-PAGE, transferred to nitrocellulose, and probed with various dilutions of plasma (A, 1:100; B, 1:200; C, 1:300; D, 1:400). Blots were developed using the ECL™ Western blotting detection system.

The ECL™ Western blotting detection system (Amersham Pharmacia Biotech, Little Chalfont, UK) is reported to be approximately 20 fold more sensitive than chromogenic detection systems and has become the method of choice to detect antibodies bound to proteins immobilized on membranes. The system produces chemiluminescence when horseradish peroxidase, conjugated to the secondary antibody, catalyses the oxidation of luminol in the presence of hydrogen peroxide. Results showed high backgrounds in certain cases, varying from patient to patient (data not shown). It has been reported that iron in the heme group of red blood cells can react with luminol, producing luminescence (Laux, 1991). It was hypothesized that hemolysis in plasma samples was responsible for the high backgrounds observed and therefore the TMB and the BCIP/NBT colorimetric detection systems (Kirkegaard and Perry Laboratories Inc.,

Gaithersburg, USA) were investigated. The TMB (3,3',5,5'-tetramethylbenzidine) detection system produces a blue colour when horseradish peroxidase, coupled to the secondary antibody, reacts with hydrogen peroxide. The BCIP/NBT (5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium) detection system produces a black/purple precipitate when alkaline phosphatase, conjugated to the secondary antibody, dephosphorylates BCIP/NBT. Comparison of the TMB, BCIP/NBT, and ECL detection systems supported the hypothesis that the high background observed with ECL was probably due the reaction between the iron group in patient plasma samples and the luminol in the ECL system (Figure 2.8). The BCIP/NBT detection system was thus chosen since this system was more sensitive and displayed decreased fading compared to the TMB system.

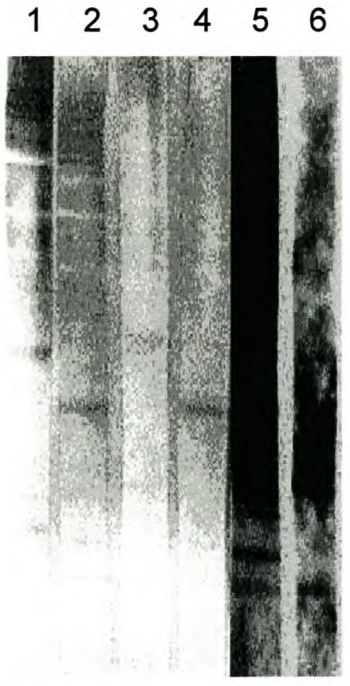

Figure 2.8 Western blot analysis using different detection systems. *M. tuberculosis* whole-cell lysate (lanes 1, 3, 5) and culture filtrate (lanes 2, 4, 6) proteins were separated by SDS-PAGE, transferred to nitrocellulose, and probed with plasma pooled from TB patients. Blots were developed using the BCIP/NBT (lanes 1, 2), TMB (lanes 3, 4) and the ECL (lanes 5, 6) detection systems.

2.4.2 Two-dimensional Western blotting

Two-dimensional (2D) Western blotting was employed to increase the resolution obtained from 1D Western blotting. Proteins were separated by 2D PAGE and transferred to nitrocellulose or PVDF (polyvinylidenedifluoride) membranes using tank electroblotting, where the gel-membrane sandwich is placed in a vertical buffer tank with platinum electrodes. To check the efficiency of transfer, gels were stained with 0.25% (w/v) Coomassie Brilliant Blue R-250 in 50% methanol and 10% acetic acid, and destained in 25% methanol and 7.5% acetic acid. Membranes were stained with 0.5% (w/v) Amido Black in 25% isopropanol and 10% acetic acid for 1 minute, and destained by several washes in distilled H_2O. Staining of gels and membranes after transfer showed suboptimal transfer. Increasing transfer time caused the transfer unit to heat up considerably, prompting us to try semi-dry blotting, where the gel-membrane sandwich is placed between horizontal graphite plates. Staining of gels and membranes after semi-dry transfer showed improved transfer efficiency, consistent with other studies reporting on this simpler, cheaper and faster method (Kyhse-Andersen, 1984). Various transfer times, buffer systems (continuous and discontinuous) and protein concentrations were investigated. Transfer at $1mA/cm^2$ for 2 hour, using the Towbin buffer system (Towbin *et al.* 1979) showed the best transfer and was used for future work (data not shown). Transfer efficiency was related to the concentration of protein separated by 2D PAGE (Figure 2.9). PVDF membranes are reported to have higher protein binding capacity and improved mechanical strength compared to nitrocellulose membranes. PVDF (Amersham Pharmacia Biotech) and nitrocellulose (S&S Protran BA85, Schleicher & Schuell Inc., Keene, USA) membranes were compared by probing with monoclonal antibodies (Figure 2. 10) or by probing with patient plasma (Figure 2.11). Transfer efficiency was better when using nitrocellulose membranes.

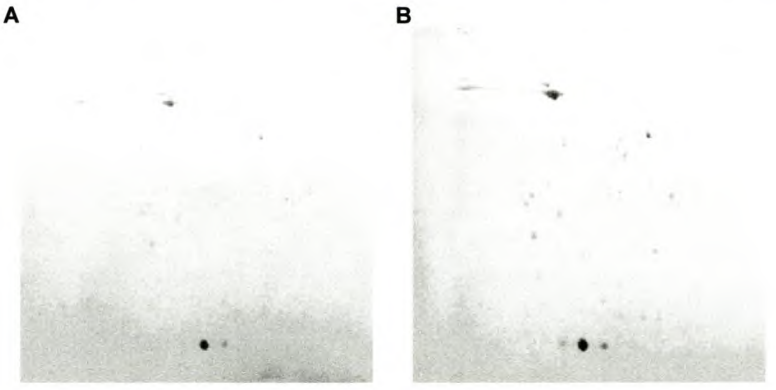

Figure 2.9 Effect of protein concentration on transfer efficiency. Thirty (A) and sixty (B) micrograms of *M. tuberculosis* H37Rv whole-cell lysate proteins was separated by 2D PAGE, transferred to PVDF, and stained with Amido Black.

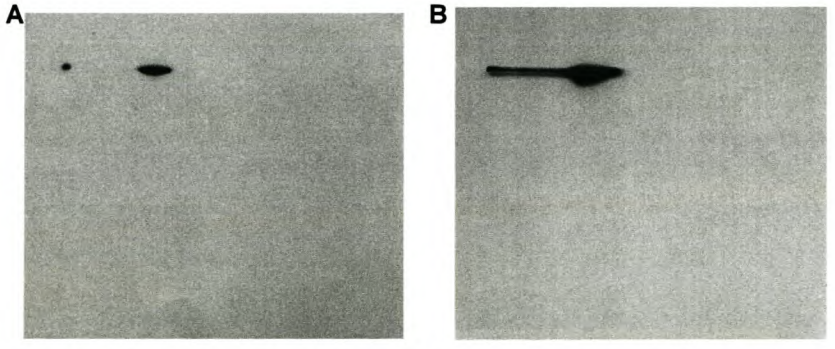

Figure 2.10 Comparison of transfer membranes using a monoclonal antibody. Sixty micrograms of *M. tuberculosis* H37Rv whole-cell lysate protein was separated by 2D PAGE, transferred to PVDF (A) or nitrocellulose (B), and probed with a mAb directed to the 65 kDa heat shock protein.

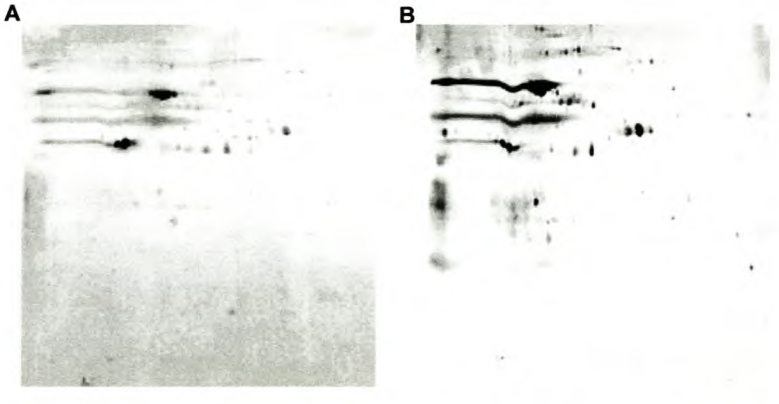

Figure 2.11 Comparison of transfer membranes using patient plasma. Sixty micrograms of *M. tuberculosis* H37Rv whole-cell lysate protein was separated by 2D PAGE, transferred to PVDF (A) or nitrocellulose (B), and probed with plasma pooled from TB patients.

2.5 Conclusions

Optimisation of the conditions described resulted in high reproducibility. Our 2D PAGE analysis was able to identify protein changes detected by others (Betts *et al.* 2002a). Similarly, ELISA and Western blot results using mAbs supplied by the National Institute of Health and the National Institute of Allergy and Infectious Diseases (Department of Microbiology, Colorado State University) were consistent with other investigators (Sonnenberg and Beslisle, 1997).

Chapter 3

PROTEIN EXPRESSION IN *MYCOBACTERIUM TUBERCULOSIS* DIFFERS ACCORDING TO GROWTH STAGE AND STRAIN TYPE

The results presented in this chapter were published as **"Protein Expression in *Mycobacterium tuberculosis* Differs According to Growth Stage and Strain Type.** Carmen Pheiffer, Joanna Betts, Pauline Lukey and Paul van Helden. **Clinical Chemistry and Laboratory Medicine**, 2002: 40, 869-875."

The style and referencing system suggested by Clinical Chemistry and Laboratory Medicine has been altered to conform to the style of the thesis.

3.1 Introduction

Response to infection by *M. tuberculosis* is heterogeneous. For example, only 5-10% of individuals infected with *M. tuberculosis* are likely to develop disease in the absence of immunosuppression (Enarson and Rouillon, 1994). Of these, 95% of patients who comply with therapy and do not harbour a drug resistant isolate are cured and fewer than 5% will "relapse" within 2 years after completion of therapy (Iseman and Sbarbaro, 1991). It is known that "relapse" can occur due to reactivation of latent infection or exogenous reinfection. In addition, in some patients *M. tuberculosis* is rapidly eradicated and patients are cured after 2-3 months of antituberculosis treatment, while in others organisms may persist for many months (Wallis *et al.* 1999).

The variability in the response to *M. tuberculosis* infection and the severity of disease may be due to differences in susceptibility of individuals, and a variety of acquired and innate host factors have been described that can change the outcome of *M. tuberculosis* infection (Bellamy and Hill, 1998b). Phenotypic differences such as induction of different immune responses of macrophages (Hoal-van Helden *et al.* 2001a, 2001b) and different rates of transmission (Warren *et al.* 2000) and adaptive immunity (Manca *et al.* 1999) have been reported for a variety of *M. tuberculosis* strains, fuelling speculation that some strains of *M. tuberculosis* are "hypervirulent". One may therefore hypothesize that this variability between strains may be due to antigenic differences which could cause variable responses in patients infected with *M. tuberculosis*, allowing certain strains to evade host defenses and cause disease in a subset of individuals, while other strains may be easily killed by those same individuals. Differential antigen expression by *M. tuberculosis* strains may explain why individuals are susceptible to

exogeneous reinfection (van Rie *et al.* 1999a) and may succumb to TB several times or may succumb to one strain type even while infected by others (Du Plessis *et al.* 2001).

Comparison between the genomes of *M. tuberculosis* H37Rv and CDC1551, a clinical isolate exhibiting a more vigorous host immune response than H37Rv (Manca *et al.* 1999), has revealed >99% identity at the nucleotide level (Delcher *et al.* 1999). Recent studies have suggested that single nucleotide substitutions are not a major source of genetic diversity within the *M. tuberculosis* complex but that insertion and deletion events could account for the perceived phenotypic differences between strains (Brosch *et al.* 2001). Using microarray analysis, Kato-Maeda *et al.* (2001) showed deletions of between 3 and 38 open reading frames (ORFs) among 15 clinical isolates when compared to H37Rv. Comparative proteomics detected seven proteins unique to CDC1551, three proteins unique to H37Rv, two proteins upregulated in H37Rv and one protein with different vertical mobility between H37Rv and CDC1551 when grown *in vitro* (Betts *et al.* 2000). However, identification of protein differences by mass spectrometry showed no obvious association with phenotype. The two-dimensional (2D) gel electrophoresis technology used in this study identified at most approximately 44% of the predicted 3924 ORFs of *M. tuberculosis* H37Rv (Cole *et al.* 1998) and the limitations of the technology may have contributed to the failure to identify many differences (Betts *et al.* 2000).

In this study we compared protein expression by *M. tuberculosis* H37Rv and two *M. tuberculosis* strains isolated from sputum specimens of TB patients residing in a community with high notification rates of TB (>1 000/100 000) in South Africa (Warren *et al.* 2000). We monitored somatic protein expression by the different strains at various stages of growth using one-dimensional (1D) sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-

PAGE). We also performed time course analysis using Western blotting and enzyme-linked immunosorbent assays (ELISAs) with a panel of mouse monoclonal antibodies (mAbs) directed to α-crystallin, the antigen 85 (Ag85) complex, PstS1, L-alanine dehydrogenase and the 65 kDa antigen (Hsp65). The 16 kDa antigen α-crystallin homologue is induced during stationary phase growth *in vitro* (Yuan *et al.* 1996) and elicits both B- and T- cell responses (Wilkinson *et al.* 1998). The antigen 85 complex (Ag85A, Ag85B and Ag85C) is a group of fibronectin-binding proteins possessing mycolyl transferase activity and all have been shown to be potent T-cell stimulants (Daffé, 2000). PstS1, a 38 kDa phosphate-binding lipoprotein, is a known B- and T-cell stimulant that has been suggested as a potential immunodiagnostic reagent (Wilkinson *et al.* 1997). Analysis of *in vitro* cultures has shown expression of L-alanine dehydrogenase in *M. tuberculosis* but not the vaccine strain *Mycobacterium bovis* bacille Calmette-Guérin (BCG) (Andersen *et al.* 1992) and the gene encoding this protein (*ald*) has been found to be upregulated during nutrient starvation of *M. tuberculosis* (Betts *et al.* 2002). Hsp65 is a highly immunoreactive heat shock protein which is conserved and expressed in all bacteria (Shinnick *et al.* 1988). Results showed that these proteins are differentially expressed according to the growth stage of the *M. tuberculosis* strain, but no major differences between strains were observed.

3.2 Materials and Methods

3.2.1 *Mycobacterium tuberculosis* strains

M. tuberculosis H37Rv was obtained from Paul Meyers (Department of Medical Microbiology, University of Cape Town, South Africa). Two clinical strains with different genotypes and frequencies in the study community were selected from the *M. tuberculosis* strain bank maintained at the University of Stellenbosch (Warren *et al.* 2000). Strain genotypes were confirmed using IS*6110* as described elsewhere (Warren *et al.* 2000) as well as by spoligotyping as described in the Isogen Bioscience BV manual (Isogen Bioscience BV, The Netherlands). All strains were fully drug sensitive.

3.2.2 Mycobacterial cultures

Mycobacterium tuberculosis strains were grown in 10 ml of Middlebrook 7H9 media (Difco, Becton Dickinson, USA) supplemented with 10% albumin-dextrose-catalase (ADC) and 0.05% Tween-80 (MADCTW). Cultures were stirred with 8 mm Teflon-coated magnetic stirrer bars at 37°C until an optical density at 600 nm (OD_{600}) >0.6 was observed. Thereafter, aliquots were frozen at –70°C. Aliquots stored at –70°C were thawed at room temperature, diluted 1:50 into 10 ml of MADCTW and grown with stirring at 37°C for 10 days. Cultures were removed from the incubator, mixed well with a 1 ml syringe, and then diluted between 1:80 and 1:200 into 20 ml of MADCTW, depending on the OD_{600} readings. Proteins were harvested after 4, 7, 14, 28 and 42 days. At each time point morphology and acid fastness was verified by Ziehl-Nielsen (ZN) staining (Heifets and Good, 1994).

3.2.3 Growth measurement

Optical densities at 600 nm (OD_{600}) were measured in a spectrophotometer (LKB Biochrom, Cambridge, UK) using 2 ml of culture. In addition, a modified protein determination method for determining bacterial growth (Meyers *et al.* 1998) was used as follows: 1 ml of culture was centrifuged at 10 000 *g* for 10 min. Pellets were washed with 1 ml of phosphate buffered saline (PBS)/1% (v/v) Tween-20 and centrifuged as before (10 000 *g*, 10 min). Pellets were frozen at –20°C until all samples were collected (all time points). Samples were thawed at room temperature, after which they were resuspended in 0.1 ml of 1 M NaOH and boiled for 10 min. Thereafter, samples were neutralised by adding 0.02 ml of 5 M HCl. Eight hundred and eighty microlitres of PBS was added, the samples were centrifuged for 30 min at 10 000 *g*, where after 800 µl of supernatant was removed for protein determination according to the formula: Protein concentration (µg/ml) = (183 X A_{230}) - (75.8 X A_{260}). All assays were performed in duplicate.

3.2.4 Extraction of mycobacterial proteins

Whole-cell extracts were prepared by harvesting two 20 ml cultures of each strain after 4, 7, 14, 28 and 42 days of growth. Cultures were pooled and pelleted by centrifugation at 1 900 *g* for 20 min. Pellets were washed twice in PBS/1% (v/v) Tween-20 and centrifuged (4 000 *g*, 5 min). Mycobacterial cells were resuspended in lysis buffer (0.3% (w/v) SDS, 200 mM DTT, 50 mM Tris-HCl pH 7.0, 1 mM PMSF and complete protease inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany)). An equal volume of Silica/Ceramic Matrix Beads (BIO 101, Vista, USA) was added, samples were mixed by vortexing and thereafter heat-killed at 80°C for 20 min prior to bead disruption in a ribolyser (BIO 101, Vista, USA). After boiling for 5 min,

samples were clarified by centrifugation at 10 000 *g* for 10 min and protein concentrations estimated using the Bradford assay (Bradford, 1976). Whole-cell lysates received from the Department of Microbiology, Colorado State University, through funds from the National Institutes of Health, National Institute of Allergy and Infectious Disease, Contract NO1-AI-75320 were used as controls.

3.2.5 Monoclonal antibodies

Mouse monoclonal antibodies (mAbs) IT-4, IT-7, IT-13, IT-23 and IT-49 raised against the 16 kDa (α-crystallin), 40 kDa (L-alanine dehydrogenase), 65 kDa (Hsp65), 38 kDa (PstS1) and 32-33 kDa (Ag85 complex) *M. tuberculosis* proteins (Engers *et al.* 1986; Khanolkar-Young *et al.* 1992; Sonnenberg and Beslisle, 1997) were made available by the Department of Microbiology, Colorado State University through funds from the National Institutes of Health, National Institute of Allergy and Infectious Disease, Contract NO1-AI-75320.

3.2.6 SDS-PAGE and Western blotting

Total cellular proteins were separated by SDS-PAGE using a 4% stack over a 12% resolving gel. Gels were stained with Coomassie Blue or proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Amersham Pharmacia Biotech, Little Chalfont, UK) and incubated with mAbs (IT-4:1:1 000, IT-7:1:2 500, IT-13:1:500, IT-23:1:100, IT-49:1:100). Blots were screened for antibody binding using horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG, CALTAG laboratories, Burlingame, USA), and bound antigens were detected using chemiluminescence detection reagents (ECL™ Western blotting detection reagents, Amersham Pharmacia Biotech, Little Chalfont, UK).

3.2.7 Enzyme-linked immunosorbent assay

Fifty microlitres of lysate sample diluted to 10 µg/ml in 0.025 M $NaHCO_3$ was allowed to bind to duplicate wells of ELISA plates (Nunc, Roskilde, Denmark) at 4°C overnight. After three washes with PBS/0.05% (v/v) Tween-20 the wells were blocked with 2% (w/v) non-fat milk powder in PBS/0.05% (v/v) Tween-20 (blocking buffer) for 2 h at 37°C. Thereafter, various dilutions of mAbs (IT-4:1:500, IT-7:1:1 000, IT-13:1:500, IT-23:1:200, IT-49:1:200) in blocking buffer were added and incubated for 2 h at 37°C. Following this, plates were washed six times with PBS/0.05% (v/v) Tween-20 and 50 µl peroxidase-conjugated goat anti-mouse IgG (CALTAG laboratories, Burlingame, USA) diluted 1:10 000 in blocking buffer was added to the wells and left for 1 h at 37°C. Plates were washed six times with PBS/0.05% (v/v) Tween-20 as before and 100 µl of tetramethylbenzidine substrate (TMB, Kirkegaard & Perry Laboratories Inc., Gaithersburg, USA) was added. After 10 min at room temperature, colour development was stopped by adding 100 µl of 1 M H_2SO_4. Plates were read at 450 nm using a bench top microplate reader (Bio-Rad laboratories, Hercules, USA).

3.3 Results

3.3.1 Strain typing

M. tuberculosis H37Rv and clinical strains that had originally been cultured from sputum specimens of TB patients were genotyped using IS*6110* and spoligotyping. DNA fingerprinting showed that *M. tuberculosis* strain 1296 and strain 1524 had 9 and 22 hybridizing copies of IS*6110*, respectively (Figure 3.1A). Fourteen copies of IS*6110* were present in *M. tuberculosis* H37Rv. Different spoligotyping patterns were observed for each of the *M. tuberculosis* strains (Figure 3.1B). Strain 1524 was classified as a Beijing family strain, because it contained only 9 spacer sequences and had a characteristic IS*6110* pattern (van Soolingen *et al.* 1995).

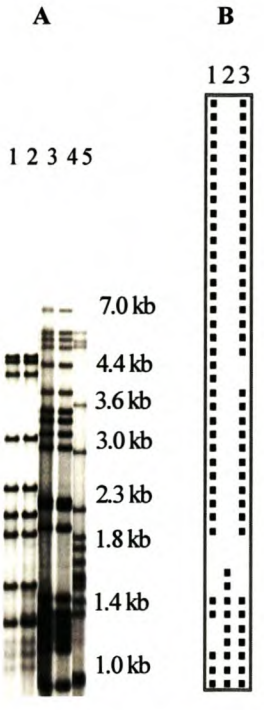

Figure 3.1 IS*6110* RFLP and spoligotype patterns of strains investigated. A. *M. tuberculosis* strain 1296 (lanes 1 and 2), strain 1524 (lanes 3 and 4) and H37Rv (lane 5) were genotyped using IS*6110* restriction fragment length polymorphism analysis. DNA fragment sizes are indicated on the right. B. Schematic representation of spoligotypes of *M. tuberculosis* strain 1296 (lane 1), strain 1524 (lane 2) and H37Rv (lane 3).

3.3.2 Growth of *M. tuberculosis*

Mycobacterial growth was analysed over a period of 42 days using OD_{600} measurements (Figure 3.2A) and a protein determination method (Meyers *et al.* 1998) (Figure 3.2B). This method is useful for heavily clumped cultures where accurate spectrophotometric measurements and colony forming units (CFU) counts are difficult or impossible. Comparison of the growth rates of the strains showed that strain 1524 initially grew slower (had a longer lag phase) but cell numbers were similar to H37Rv and strain 1296 at day 42. The two methods of growth determination correlated well. ZN staining of cultures showed no contamination during growth. Although ZN staining is not quantitative, ZN results appeared to correlate with growth as determined by OD_{600} and protein determination (data not shown).

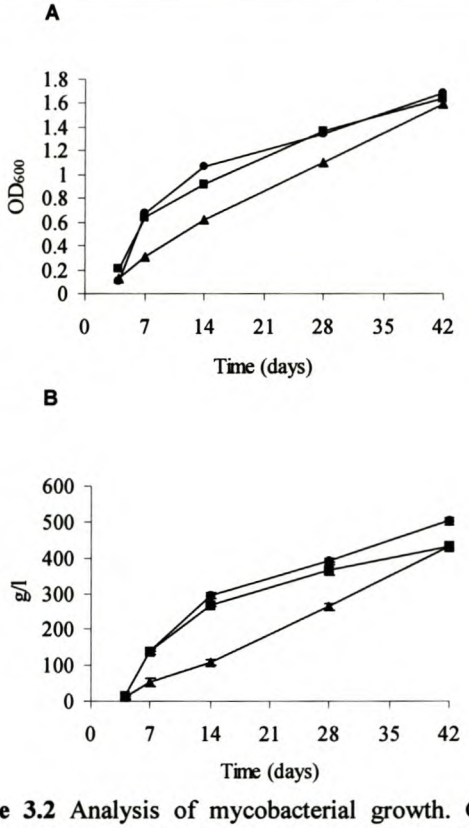

Figure 3.2 Analysis of mycobacterial growth. Growth of *M. tuberculosis* strain 1296 (■), strain 1524 (▲) and H37Rv (•) in Middlebrook 7H9 medium was analysed using OD_{600} (A) and a protein determination method (B). Each data point represents the average of two measurements. Error bars show the standard deviations from the mean.

3.3.3 Analysis of protein expression by different *M. tuberculosis* strains during *in vitro* growth

Protein expression by strains 1296, 1524 and H37Rv was analysed by 1D SDS-PAGE at various time points during growth (day 4, 7, 14, 28 and 42). Comparison of the different *M. tuberculosis* extracts (Figure 3.3) revealed mainly quantitative differences that appeared to be growth-, rather than strain-dependent. The most prominent bands ranged in size from 16 kDa to 66 kDa. Major variants at approximately 10 kDa, 16 kDa, 24 kDa, 26 kDa, 45 kDa, 60 kDa and 65 kDa are indicated by arrows in Figure 3.3. The fewer bands observed at day 28 and day 42 could be due to shut-down of protein synthesis or protein degradation later in the culture period. Our results illustrate modulation of *M. tuberculosis* proteins during growth, even with a relatively crude measurement tool.

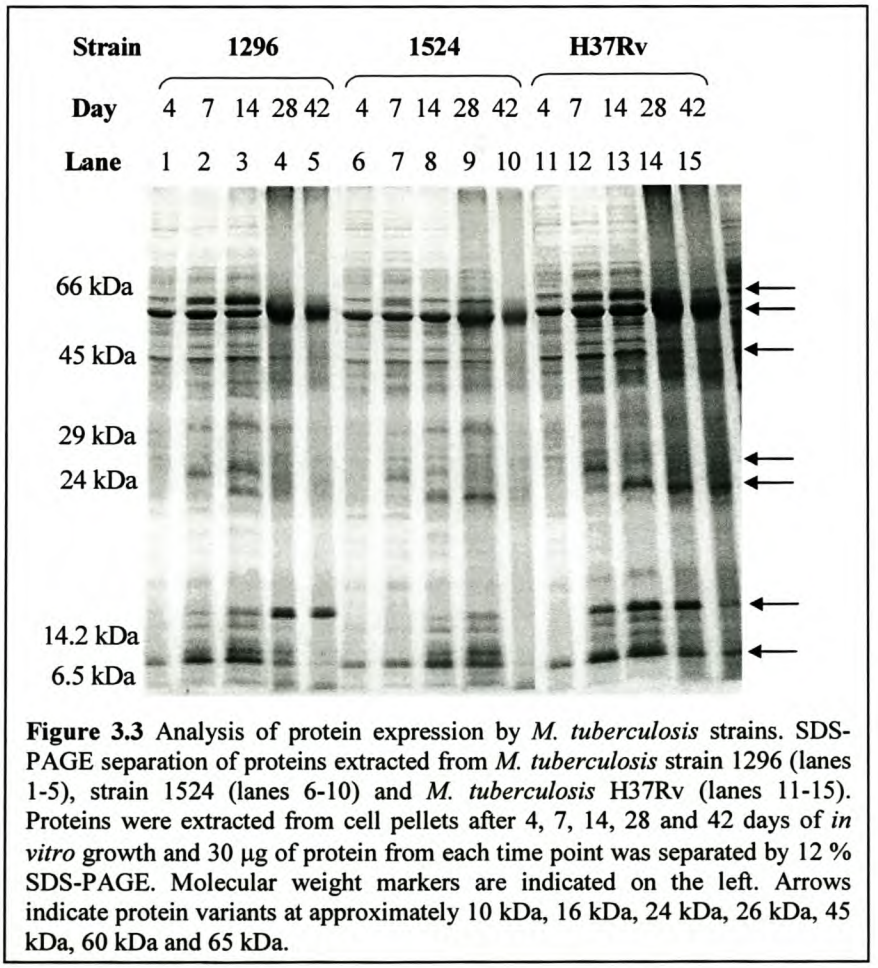

Figure 3.3 Analysis of protein expression by *M. tuberculosis* strains. SDS-PAGE separation of proteins extracted from *M. tuberculosis* strain 1296 (lanes 1-5), strain 1524 (lanes 6-10) and *M. tuberculosis* H37Rv (lanes 11-15). Proteins were extracted from cell pellets after 4, 7, 14, 28 and 42 days of *in vitro* growth and 30 µg of protein from each time point was separated by 12 % SDS-PAGE. Molecular weight markers are indicated on the left. Arrows indicate protein variants at approximately 10 kDa, 16 kDa, 24 kDa, 26 kDa, 45 kDa, 60 kDa and 65 kDa.

3.3.4 Comparison of expression of proteins reactive with mAbs during growth of different *M. tuberculosis* strains

Total proteins harvested from the different *M. tuberculosis* strains at the various time points were probed with a panel of five mAbs to further characterize the differentially expressed proteins observed by SDS-PAGE. Western blot analysis showed that varying levels of the proteins to which the mAbs react are produced during different growth stages of the *M. tuberculosis* strains, as indicated in Figure 3.4. Although differences were noted, protein expression in H37Rv and strain 1296 were essentially similar. Levels of α-crystallin generally increased over the time course, consistent with previous reports of it being a stationary phase associated protein (Yuan *et al.* 1996). Conversely, levels of Hsp65 decreased across the time course, with the lowest levels observed at day 42. PstS1 levels peaked between day 14 and day 28 and then declined. Strain 1524 showed similar patterns of Hsp65 and Ag85 complex expression to the other strains but very little α-crystallin (except at day 28 and day 42) and PstS1 were detected. The Ag85 complex band appeared as a doublet, probably corresponding to Ag85A and Ag85B (Figure 3.4B). Most of the differences between the three strains were observed for L-alanine dehydrogenase expression.

Monoclonal antibody IT-7 reacted with two products, the expected band of 40 kDa and a band of 16 kDa. This is in agreement with Sonnenberg and Beslisle (Sonnenberg and Beslisle, 1997) who also reported that this mAb reacted with a band of 16 kDa. Of interest is the increased size of the 40 kDa antigen in H37Rv in day 7 and day 14 extracts (Figure 3.4D). This could be due to protein modifications such as phosphorylation or glycosylation. Furthermore additional

bands, probably due to protein degradation, were observed below the expected bands of 40 kDa and 16 kDa in the day 42 extract of strain 1296.

ELISA essentially corroborated Western blot results, with α-crystallin, PstS1, L-alanine dehydrogenase and Hsp65 expression varying during growth of the *M. tuberculosis* strains (data not shown). Again levels of α-crystallin, PstS1 and L-alanine dehydrogenase were lower for strain 1524 than the other strains. However, the Ag85 complex was not detected in any of the strains by ELISA.

Our results show that although expression of the antigens investigated differed quantitatively during growth, expression seemed to be conserved in the strains investigated. Reactivity of the mAbs with our H37Rv whole-cell lysate proteins was comparable to that of whole-cell lysates received from the NIH in both Western blots and ELISAs (data not shown).

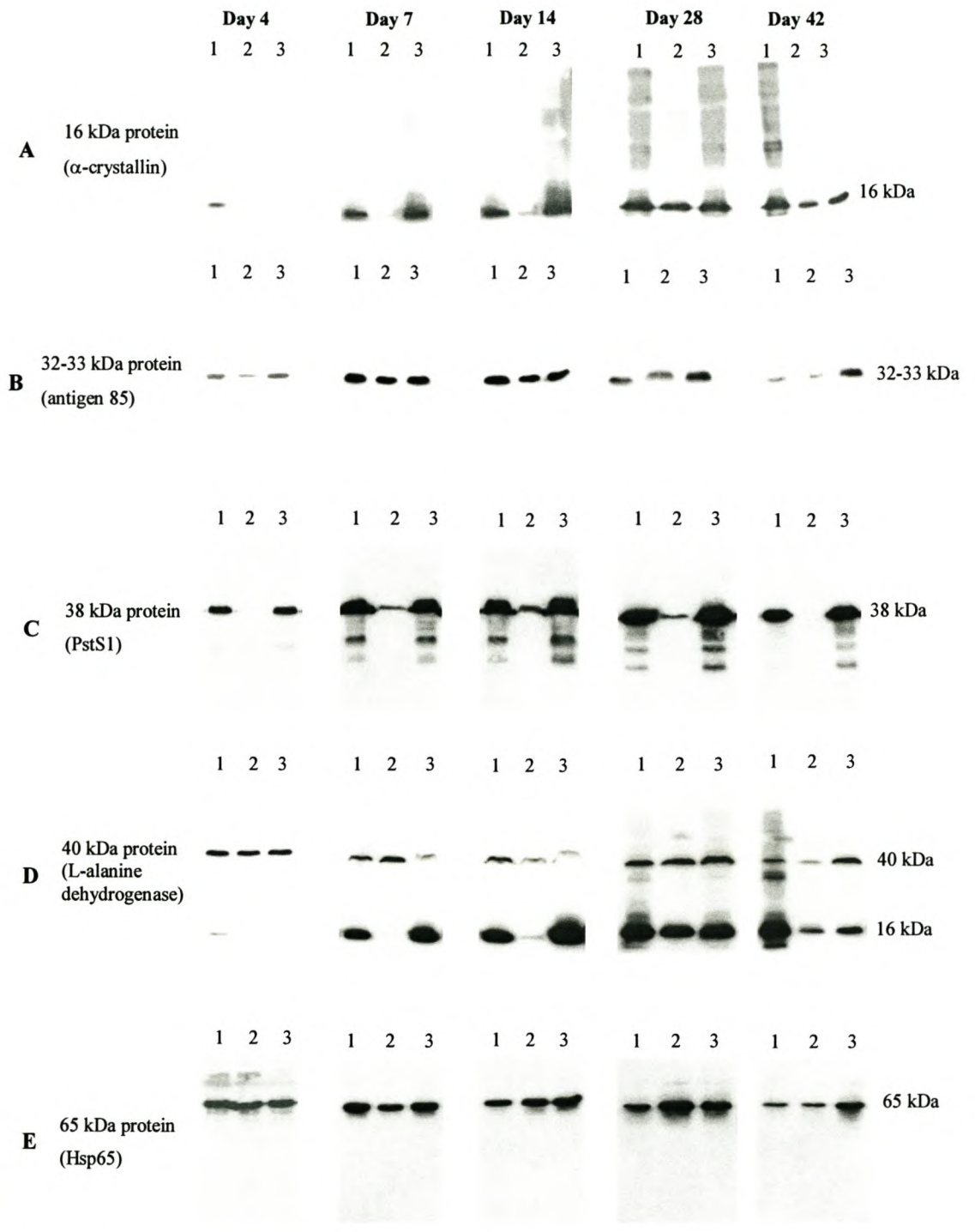

Figure 3.4 Western blot analysis. Proteins extracted from *M. tuberculosis* strain 1296 (lane 1), strain 1524 (lane 2) and H37Rv (lane 3) were probed with monoclonal antibodies (mAbs) directed to the 16 kDa (α-crystallin) protein (A), the 32-33 kDa (Ag85 complex) protein (B), the 38 kDa (PstS1) protein (C), the 40 kDa (L-alanine dehydrogenase) protein (D) and the 65 kDa (Hsp65) protein (E). Proteins were extracted from cell pellets after 4, 7, 14, 28 and 42 days of *in vitro* growth and 10 µg separated by 12% SDS-PAGE. After transfer onto PVDF membranes, proteins were probed with various dilutions of mAbs as described before.

3.4 Discussion

Recent studies have suggested that phenotypic differences between strains can possibly contribute to different outcomes after *M. tuberculosis* infection (Hoal-van Helden *et al.* 2001a, 2001b). Furthermore, differential antigen expression by *M. tuberculosis* strains may potentially explain why certain patients are susceptible to a repeat episode of TB, particularly from a different strain. In this study we compared protein expression by *M. tuberculosis* H37Rv and two clinical isolates to determine whether differential antigen expression contributes to the different phenotypes expressed by these strains.

DNA fingerprinting identified strain 1296 and strain 1524 as belonging to family 23 (9 hybridising copies of IS*6110*) and 29 (22 hybridising copies of IS*6110*), respectively. Epidemiological studies in our community have shown that the frequency of family 29 isolates is >10 times that of family 23 strains (Warren *et al.* 2000). Furthermore, spoligotyping revealed that strain 1524 (family 29) belongs to the Beijing family of strains, speculated to have originated in Asia (van Soolingen *et al.* 1995) and reported to be responsible for most of the outbreaks of drug resistance in our community (van Rie *et al.* 1999b).

Comparison of growth over a time course of 42 days showed no significant difference in growth rate of the *M. tuberculosis* strains, suggesting that growth rate of mycobacteria *in vitro* is not an indicator of transmissibility. Our findings are in agreement with others (Betts *et al.* 2000; Manca *et al.* 1999) who also failed to show differences in growth rates of *M. tuberculosis* H37Rv, CDC1551 and two clinical isolates when grown *in vitro* and *in vivo*.

SDS-PAGE and Coomassie staining of total cellular proteins obtained from *M. tuberculosis* H37Rv and the two clinical isolates showed that protein profiles changed during growth in culture. *M. tuberculosis* H37Rv has 3924 predicted genes (Cole *et al.* 1998) and although 1D (or even 2D) gel electrophoresis will not enable all the expressed proteins to be visualised, we were able to show that variation of the major proteins identified appeared to be growth-stage, rather than strain-dependent. The genomes of *M. tuberculosis* strains are relatively stable (Musser *et al.* 2000) and our results are in agreement with previous studies showing few differences between *M. tuberculosis* strains at the proteome level (Betts *et al.* 2000).

ELISAs and Western blot analysis confirmed our findings using SDS-PAGE, where variation of protein expression appeared to be largely independent of *M. tuberculosis* strain analysed but showed modulation during growth. Although variable levels were expressed during growth, all strains investigated expressed α-crystallin, PstS1, L-alanine dehydrogenase and Hsp65 to some extent. Interestingly, strain 1524 expressed lower levels of α-crystallin, PstS1 and L-alanine dehydrogenase compared to the other strains. The least variation between strains was observed with Hsp65. No Ag85 expression was detected by ELISA, whereas Western blot analysis showed Ag85 expression in all strains. Using ELISA we have been able to detect Ag85 expression within a culture filtrate protein fraction (data not shown), suggesting that levels of these proteins are low in the whole-cell lysates of the strains studied and that Western blot detection is simply more sensitive than ELISA. PVDF membranes have greater protein binding capacity than microtitre plates and, for Western blot detection, proteins are separated and more total protein can be applied. Furthermore, mycobacteria are rich in lipids which may compete with proteins in the ELISA assay. Slight conformational differences in the antigen when bound to

the ELISA plate compared to the PVDF, causing partial obstruction of the epitope may also explain the differences observed between ELISA and Western blotting.

Our results suggest that the antigen repertoire presented to the host immune response may be quantitatively dependent on the growth stage of the infecting *M. tuberculosis* strain. This implies that the profile of antigens presented to the host immune response may be affected by disease progression, as previously suggested by Samanich *et al.* (1998). In addition, although the same antigens may be expressed in all strains, a quantitative difference may also evoke considerable differences in the host response. This could possibly explain some of the heterogeneous humoral immune response observed in TB patients and why no fully effective serodiagnostic has been developed to date (Lyashchenko *et al.* 1998a). These results suggest that antigens that are not susceptible to growth phase variation should be used for development of serodiagnostic tests. It should be noted, however, that antigenic differences between strains may be more pronounced in culture filtrates, where the secreted proteins are found (Andersen *et al.* 1991) and therefore these should be investigated. Future studies in our laboratory are being directed to analysis of secreted proteins using 2D gel electrophoresis.

Although protein expression varied with growth stage of the strains, all strains expressed the antigens analysed at some time, suggesting that differential antigen expression may not explain why certain patients are susceptible to a repeat episode of TB (van Rie *et al.* 1999a). *M. tuberculosis* may adapt to different host environments (within macrophages, granulomas, necrotic lesions, etc.) by adopting differential gene expression strategies (Cappelli *et al.* 2001). Our current studies are focused on comparing antigen expression by different *M. tuberculosis* strains when grown under environmental conditions that more closely mimic the host environment.

Chapter 4

SERODIAGNOSIS OF TUBERCULOSIS COMPLICATED BY VARIABLE PROTEIN EXPRESSION OF *MYCOBACTERIUM TUBERCULOSIS* STRAINS AND THE HETEROGENEOUS HOST HUMORAL IMMUNE RESPONSE

The results presented in this chapter have been submitted to the **Journal of Clinical and Diagnostic Laboratory Immunology** as "**Serodiagnosis of tuberculosis complicated by variable protein expression of *Mycobacterium tuberculosis* strains and the heterogeneous host humoral immune response.** Carmen Pheiffer, Joanna Betts, Pauline Lukey and Paul van Helden" and is currently under review.

The style and referencing system suggested by Clinical and Diagnostic Laboratory Immunology has been altered to conform to the style of the thesis.

4.1 Introduction

The need to develop a faster, easier, more sensitive but economical method to diagnose tuberculosis (TB), especially smear negative pulmonary TB, extrapulmonary TB and childhood TB (Bothamley, 1995; Chan *et al.* 2000), has sparked interest in serodiagnosis, particularly in developing countries where the greatest proportion of the world's TB burden lies (Dye *et al.* 1999). Although many potential tests have been developed, no test has shown satisfactory sensitivity and specificity for general clinical use (Chan *et al.* 2000). The failure of serology for acceptance for TB diagnosis is thought to be mainly due to the heterogeneous host humoral immune responses of TB patients (Lyashchenko *et al.* 1998a). Several factors, including host genetics (Bothamley *et al.* 1989), geographical location (Daniel and Debanne, 1987) and extent of disease (Samanich *et al.* 1998) can contribute to the humoral heterogeneity observed amongst TB patients.

Studies have suggested that *Mycobacterium tuberculosis* strain-related differences may also influence the host antibody response (Lyashchenko *et al.* 1998a; Samanich *et al.* 2001). Identification of proteins differentially expressed by different *M. tuberculosis* strains may shed some light on the reasons for the heterogeneous host humoral immune responses of TB patients, as antigenic diversity due to differential protein expression has been reported for a number of pathogens, including bacteria, viruses, helminths and protozoa (Smith *et al.* 2002). Furthermore, it has been speculated that strains belonging to the Beijing family, a family of *M. tuberculosis* strains distributed worldwide and often associated with drug resistance (Glynn *et al.* 2002), have a genetic advantage and that the dominance of such families, compared to other less prevalent clinical isolates, may be related to differential protein expression (Bifani *et al.* 2002).

Previously, proteomics has been used to compare protein expression of the *M. tuberculosis* strains H37Rv and Erdman to the vaccine strain *Mycobacterium bovis* Bacille Calmette-Guérin (BCG) in an attempt to identify proteins contributing to the pathogenesis of *M. tuberculosis* (Jungblut *et al.* 1999). Comparative proteomics of *M. tuberculosis* H37Rv and the clinical strain CDC1551, believed to elicit a more vigorous host immune response (Manca *et al.* 1999), detected several protein differences between the strains (Betts *et al.* 2000). A more recent study showed differential expression of the PE-PGRS proteins, speculated to be variable surface antigens, between clinical isolates of *M. tuberculosis* (Banu *et al.* 2002).

In a previous study we compared protein expression of two *M. tuberculosis* clinical strains, originally isolated from patients from a community in Cape Town, South Africa with a very high TB incidence (Warren *et al.* 2000), to the laboratory strain H37Rv using one-dimensional (1D) polyacrylamide gel electrophoresis (PAGE), enzyme-linked immunosorbent assay (ELISA) and Western blotting (Pheiffer *et al.* 2002). Based on the number of IS*6110* insertions and spoligotyping, the two clinical strains were classified as belonging to the Beijing family and family 23, respectively (Pheiffer *et al.* 2002). Between 1993 and 1998, 18.7% of TB cases in this community were reported to be due to infection with Beijing strains, whereas family 23 strains were responsible for only 1.4% of TB cases (Warren *et al.* 2000). Twenty eight strain families have been identified in this community (van Helden *et al.* 2002), with the prevalence of Beijing strains being exceeded only by *M. tuberculosis* strains belonging to family 11, which were responsible for 24.5% of TB cases during 1993-1998. Results from a previous study (Pheiffer *et al.* 2002) showed that protein expression differences between strains could be growth-phase dependent, although some differences between the strains were observed. However due to the low resolving power of 1D gel electrophoresis, these differences were not identifiable.

Here, we have extended our previous study through the use of two-dimensional (2D) PAGE coupled with identification of protein spot differences by mass spectrometry and Western blot analysis of the expression of 14 *M. tuberculosis* antigens. Furthermore we have used plasma from patients infected with Beijing, family 23 and family 11 strains to investigate differences in antigen recognition. This study has revealed considerable differences in protein expression between the three strains, particularly between the two clinical strains compared to H37Rv. Careful analysis of these differences and the differences in the antibody recognition profiles could provide us with clues to explain the heterogeneous host humoral immune response and also the different frequencies of the clinical strains in our study population, where the Beijing strain is at least ten fold more frequent than the family 23 strain (Warren *et al.* 2000).

4.2 Materials and Methods

4.2.1 *Mycobacterium tuberculosis* strains and culture

M. tuberculosis H37Rv (ATCC 25618) and two local clinical strains (Beijing (SAWC 1524) and family 23 (SAWC 1296)) were grown in modified Sauton medium (Andersen *et al. 1991*). Briefly, mycobacterial strains at late exponential growth (OD_{600}>0.6) were diluted 1:10 into 200 ml of Sauton medium and grown at 37°C. After four weeks, cultures were harvested by centrifugation (1 900 *g*, 20 min) and mycobacterial pellets were washed twice with PBS containing 1% (v/v) Tween-20. Proteins used in Western blots with plasma from TB patients were prepared from strains during logarithmic growth in Middlebrook 7H9 media (Difco, Becton Dickinson, USA) supplemented with 10% (v/v) albumin-dextrose-catalase and 0.05% (v/v) Tween-80 as previously described (Pheiffer *et al.* 2002). Morphology and acid-fastness was checked by Ziehl-Nielsen staining (Heifets and Good, 1994). All strains were fully sensitive to isoniazid, rifampicin and ethambutol.

4.2.2 Protein extraction

Whole-cell lysate (WCL) proteins were extracted by bead disruption in lysis buffer (0.3% (w/v) SDS, 200 mM DTT, 50 mM Tris-HCl pH 7.0, 1 mM PMSF and complete protease inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany)) as previously described (Pheiffer *et al.* 2002). Culture filtrate proteins (CFP) were prepared from culture supernatants sterilized by sequential filtration through 0.45 μm and 0.22 μm filter units (Corning, New York, USA). Filtrates were concentrated using Centricon Plus-80 filter units (Biomax-PB membrane, 5000 MWCO), followed by Centricon units (YM membrane, 3000 MWCO) (Amicon

Bioseparations, Millipore Corporation, Bedford, USA). WCL and CFP concentrations were estimated using the Bradford assay (Bradford, 1976).

4.2.3 Two-dimensional gel electrophoresis

Proteins were separated by 2D PAGE as previously described (Betts *et al.* 2000), with minor modifications. Briefly, 20 µg (60 µg for Western blotting) of protein was resuspended in rehydration buffer (8 M urea, 2% (w/v) CHAPS, 10 mM DTT, 2% (v/v) immobilized pH gradient (IPG) buffer (pH 4-7), trace bromophenol blue) and applied to pH 4-7 IPG strips (Amersham Biosciences, Uppsala, Sweden) for overnight rehydration. Isoelectric focusing was performed using a Multiphor II system (Amersham Biosciences) as follows: 100 V for 2 h, 300 V for 2 h, 1000 V for 1 h, 3500 V for 20 h (3500 V for 27.5 h for Western blotting). Second dimension separation was carried out by placing equilibrated IPG strips (Bjellqvist *et al.* 1993, http://www.expasy.ch/ch2d/protocols/) onto 12% PAGE gels and sealing with 0.5% (w/v) agarose in cathode buffer (defined below), containing a trace amount of bromophenol blue. Anode (375 mM Tris-HCl, pH 8.8) and cathode (192 M glycine, 0.1% (w/v) SDS, pH 8.3) buffers as described by Herbert *et al.* (Herbert *et al.* 1998) were used. Proteins were visualized by silver staining using an ammoniacal stain (Bjellqvist *et al.* 1993; http://www.expasy.ch/ch2d/protocols/). Gels were air dried (BioRad GelAir Dryer, Hercules, USA) and compared by visualization on a light box. When protein spot differences were noted, gels were rerun, stained with a silver stain compatible with mass spectrometry (Betts and Smith, 2001) and spots of interest excised from the gel.

4.2.4 Sample preparation for mass spectrometry

Gel pieces excised from the gel were washed with dd-H_20 for 10 min, followed by 100% acetonitrile for 5 min, centrifuged and then dehydrated by vacuum centrifugation. Automated robotic digestion was carried out using a MassPREP station (Micromass, Manchester, UK), equipped with four probes for aspirating and dispensing reagent and washing solutions and a heated incubation platform. Samples were destained with 50 mM ammonium bicarbonate: acetonitrile (1:1), then reduced and alkylated with 10 mM DTT and 55 mM iodoacetamide respectively. This was followed by in-gel digestion with porcine trypsin (sequencing grade, Promega, Madison, WI, USA) (6 ng/ul) in 50 mM ammonium bicarbonate (25 µl) for 5 h at 37°C. The resulting peptides were then extracted with 1% formic acid (v/v): acetonitrile (98:2).

4.2.5 Nanoscale liquid chromatography tandem mass spectrometry (LC/MS/MS) and database searching

Samples were introduced using the Micromass CapLC system (Micromass, Manchester, UK), comprising a low flow capillary HPLC pump and autosampler. A 10 port valve was configured with a pre-concentration column (300 µm ID x 5 mm C18 PepMap, LC Packings, Amsterdam, NL) and a nanoscale analytical column (75 µm ID x 15 cm C18 PepMap, LC Packings, Amsterdam, NL). Peptides were eluted using a reverse phase gradient of 5-50% buffer B over 30 min (A= 5% (v/v) acetonitrile, 0.1% (v/v) formic acid; B= 95% (v/v) acetonitrile, 0.1% (v/v) formic acid) at a flow rate of approximately 200 nl/min. All data were acquired using a Q-Tof Ultima API (Micromass, Manchester, UK) hybrid quadrupole orthogonal acceleration time of flight mass spectrometer equipped with a nanospray source. Up to 8 precursor ions were automatically selected from the TOF/MS survey scan for MS/MS per cycle, and collision

energies were chosen automatically based on the m/z value and the charge state of the selected precursor ions. Peptide sequence data generated was searched against the non-redundant protein database using MASCOT (http://www.matrixscience.com). Search parameters included the fixed modification carboamidomethyl, due to the alkylation of cysteine residues by iodoacetamide and the variable modification, oxidation of methionine residues.

4.2.6 Monoclonal antibodies

Mouse monoclonal antibodies (mAbs) IT-4, IT-7, IT-13, 1T-19, IT-23, IT-38, IT-42, IT-43, IT-49, IT-51, IT-52, IT-53 and IT-58 raised against the 16 kDa (α-crystallin), 40 kDa (L-alanine dehydrogenase), 65 kDa (Hsp65), 19 kDa, 38 kDa (PstS1), 20 kDa, 82 kDa (KatG), 56 kDa, 32-33 kDa (Ag85 complex), 17 kDa, 25 kDa (MPT51), 96 kDa and 47 kDa *M. tuberculosis* proteins (Engers *et al.* 1986; Khanolkar-Young *et al.* 1992; Sonnenberg and Beslisle, 1997) respectively were made available by the Department of Microbiology, Colorado State University through funds from the National Institutes of Health, National Institute of Allergy and Infectious Disease, Contract NO1-AI-75320. HYB76-8 directed to ESAT6 (Sorensen *et al.* 1995) was received from Karin Weldingh (Statens Serum Institut, Copenhagen, Denmark).

4.2.7 One-dimensional gel electrophoresis and Western blotting

Proteins were separated by SDS-PAGE using a 4% stack over a 12% resolving gel and transferred to PVDF membranes (Amersham Biosciences) by tank blotting. Blots were incubated with mAbs (IT-4, 1:1000; IT-7, 1:2500; IT-13, 1:500; IT-19, 1:100; IT-23, 1:100; IT-38, 1:100; IT-42, 1:100; IT-43, 1:100; IT-49, 1:100; IT-51, 1:100; IT-52, 1:100; IT-53, 1:100; IT-58, 1:100; HYB76-8, 1:100) and plasma (1:100 and 1:500) and screened for antibody binding with a

horseradish peroxidase-conjugated goat anti-mouse IgG (1:10 000, CALTAG laboratories, Burlingame, USA) as the secondary antibody for mAbs and an alkaline phosphatase-conjugated goat anti-human IgG (1:2 000, Kirkegaard and Perry Laboratories Inc., Gaithersburg, USA) for plasma. Bound antigens were detected using chemiluminescence detection reagents (ECL™ Western blotting detection reagents, Amersham Biosciences) or with the 5-bromo-4-chloro-indolyl-phosphatase (BCIP)/nitroblue tetrazolium (NBT) substrate (Kirkegaard & Perry Laboratories Inc.) for plasma samples.

4.2.8 Two-dimensional Western blotting

For Western blotting, 2D gels were transferred to nitrocellulose membranes (S&S Protran BA85, Schleicher & Schuell Inc., Keene, USA) using semi-dry blotting. Blots obtained from 2D gels were probed with mAbs IT-4 and IT-23 using the same conditions as for 1D Western blotting. For blots using plasma from TB patients, plasma was diluted 1:150 and screened for antibody binding using alkaline phosphatase-conjugated goat anti-human IgG (Kirkegaard & Perry Laboratories Inc.) as previously described. Immunoreactive antigens were detected using the BCIP/NBT substrate (Kirkegaard & Perry Laboratories Inc.) as described above.

4.2.9 Patients

TB patients used in this study were culture positive for *M. tuberculosis* drug sensitive organisms. The smear status, disease episode, anti-tuberculosis chemotherapy status and IS*6110* genotype of the infecting *M. tuberculosis* strain was documented for all patient samples (Table 4.1). Blood was collected by clinical staff, plasma collected by centrifugation at 2 500 *g* for 5 minutes, and stored at -20°C. Ethical approval for this study was obtained from the University of

Stellenbosch Faculty of Health Sciences ethics committee and samples were given only after informed consent.

4.2.10 PCR amplification and DNA sequence analysis

Primers were designed to amplify approximately 550 bp 5'and 150 bp 3' from the start codon of the *pstS1* and *katG* genes of *M. tuberculosis*. PCR reactions were performed with HotstarTaq™ DNA polymerase (Qiagen GmbH, Hilden, Germany). Reaction mixtures contained approximately 500 µg DNA template, 50 pmol of each primer, 1 X Reaction Buffer, and 1 U enzyme. Cycling conditions included a 15 min 95°C denaturation step, followed by 35 cycles of 94°C for 30 s, 1 min at 56°C for *pstS1* and 61°C for *katG*, followed by a 7 min extension step at 72°C. PCR products were electrophoretically fractionated in a 1% (w/v) agarose gel, then visualized under UV after ethidium bromide staining. PCR products were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega) and sequenced on an ABI3100 automatic sequencer. DNA sequences obtained were blasted against the published H37Rv sequence (http://genolist.pasteur.fr/TubercuList/) using DNAman (DNAMAN Version 4.1).

Table 4.1 TB patient data

Patient number	Strain family[a]	Smear status[b]	Anti-TB treatment[c]	Culture positive[d]	Disease episode[e]
97	11	positive	yes (6 months)	yes	second
484	11	negative	no	yes	first
522	11	negative	no	yes	first
672	11	positive	no	yes	first
726	23	negative	yes (4 days)	yes	first
880	29 / Beijing	positive	no	yes	first
881	29 / Beijing	positive	no	yes	first
941	29 / Beijing	positive	no	yes	first

[a]*M. tuberculosis* strains are grouped into families based on the number of IS*6110* insertion elements identified by DNA fingerprinting. The strain family of the *M. tuberculosis* strain isolated from the patient sputum is shown.

[b]Smear status refers to whether a patient's sputum stained positive or negative for acid-fast bacilli.

[c]Anti-TB treatment refers to the six month TB treatment regimen prescribed by DOTS (isoniazid, pyrazinamide, rifampican, streptomycin). The time blood was taken after the commencement of treatment is shown.

[d]Culture positive refers to the ability to culture *M. tuberculosis* from patient sputum.

[e]Disease episode refers to the TB episode the patient was experiencing.

4.3 Results

4.3.1 2D PAGE and protein identification

Mycobacterial strains were cultured in modified Sauton medium, a synthetic medium without protein enrichment (Andersen *et al.* 1991) for four weeks. This enabled culture filtrate proteins to be analysed free of BSA contamination. Pilot studies showed that after four weeks growth all three strains were in late logarithmic growth, thereby minimising growth-related differences between strains. Whole-cell lysate and culture filtrate proteins were separated by 2D PAGE using pH 4-7 IPG strips and 12% PAGE gels. Pilot experiments demonstrated that many *M. tuberculosis* proteins focus between pH 4 and 7 and the best resolution for comparison was achieved using this pH range. The 2D gels of whole-cell lysate and culture filtrate proteins derived from *M. tuberculosis* H37Rv, the Beijing strain and the family 23 strain are shown in Figure 4.1. The 2D maps were highly similar between the strains, enabling visual comparison. Spot differences consistent between quadruplicate gels and obvious by visual examination were analysed further. Protein spots of interest were analysed by liquid chromatography tandem mass spectrometry (LC/MS/MS). All proteins identified had their counterparts in all three strains, although quantitative expression differences were observed. Eleven spot differences were observed between H37Rv and the clinical strains. Protein spots identified by LC/MS/MS are indicated in Figure 4.1 and their identities listed in Table 4.2. Eight protein spots were increased in the whole-cell lysates of both clinical isolates relative to H37Rv. Seven of these were identified as α-crystallin and two proteins, Rv2005c and the 35 kDa antigen were identified within the eighth spot. Previous studies have also reported the appearance of multiple α-crystallin species with different molecular weights and isoelectric points (Betts *et al.* 2000; Sonnenberg and Beslisle, 1997). One of the factors contributing to mobility variants of α-crystallin may be

oxidation of methionine residues as recently demonstrated by Abulimiti *et al.* (Abulimiti *et al.* 2003). Three spots were decreased in the culture filtrates of both clinical strains compared to H37Rv. Ag85A was identified in each of these spots. However, spot 9 also contained Rv1096 and spot 10 three other proteins; Ag85C, Rv0831c and CysA3. One spot, identified as containing both PstS1 and Ag85B was decreased in culture filtrates of the Beijing strain compared to both H37Rv and the family 23 strain.

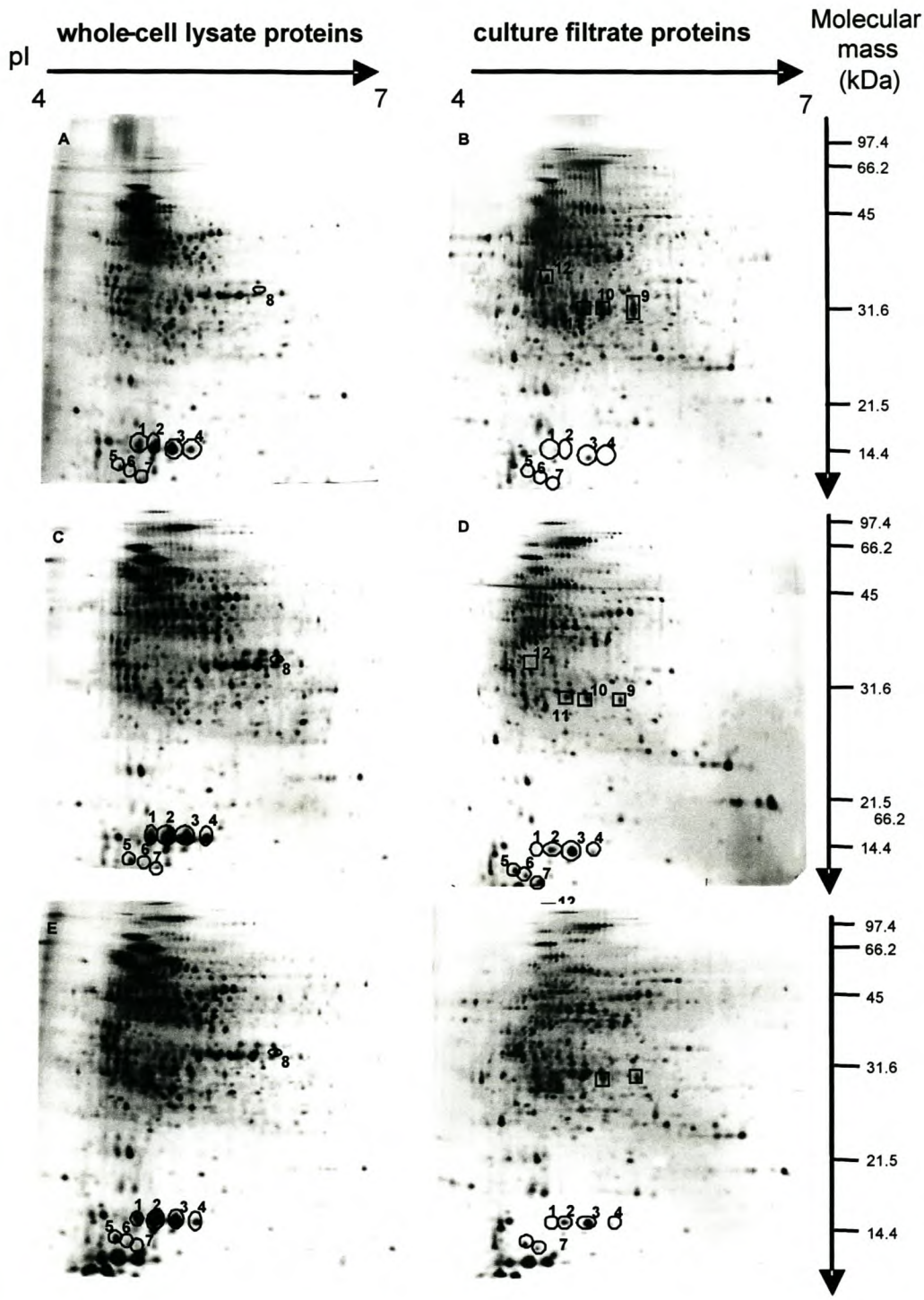

Figure 4.1 2D gels of whole-cell lysate (WCL) and culture filtrate proteins (CFP) extracted from the three *M. tuberculosis* strains. (A) H37Rv, WCL; (B) H37Rv, CFP; (C) Beijing family strain, WCL; (D) Beijing family strain, CFP; (E) family 23 strain, WCL; (F) family 23 strain, CFP. Protein spots with increased intensity in clinical strains are ringed and those with increased intensity in H37Rv are boxed. Their identities are listed in Table 4.2.

Table 4.2 Protein spot differences observed for whole-cell lysate and culture filtrate fractions of *M. tuberculosis* H37Rv, the Beijing strain and the family 23 strain.

Spot	Fraction	ORF	Gene Name	Protein	Matched peptides	Mw[a]	pI[a]
Increased expression in both clinical strains							
1-7	WCL/CF	Rv2031c	*hspX*	heat shock protein, HspX	SEFAYGSFVR TVSLPVGADEDDIK	16096	5.00
8	WCL	Rv2005c	-	conserved hypothetical protein	YANAIGSAELAESSVQGR	30985	5.53
		Rv2744c	*35kd_ag*	conserved 35 kDa alanine-rich protein	GLLGSVSSSLVR	29257	5.71
Decreased expression in both clinical strains							
9	CF	Rv1096[b]	-	possible glycosyl hydrolase	ANDVIAAATGR LVTVSELLGPR	31109	6.52
		Rv3804c	*fbpA*	antigen 85A	FLEGFVR ASDMWGPK NDPLLNVGK ALGATPNTGPAPQGA	35686	6.08
10	CF	Rv0129c	*fbpC*	antigen 85C	NDPMVQIPR EMPAWLQANK	36771	5.92
		Rv0831c[b]	-	conserved hypothetical protein	NQAIVVETTAYR	33885	5.88
		Rv3117[b]	*cysA3*	probable thiosulphate sulphurtransferase	DFVDAQQFSK	31014	5.14
		Rv3804c	*fbpA*	antigen 85A	NDPLLNVGK ALGATPNTGPAPQGA	35686	6.08
11	CF	Rv3804c	*fbpA*	antigen 85A	FLEGFVR NDPLLNVGK ALGATPNTGPAPQGA	35686	6.08
Decreased expression in Beijing strain only							
12	CF	Rv0934	*pstS1*	Periplasmic phosphate-binding lipoprotein	VLAAMYQGTIK	38243	5.14
		Rv1886c	*fbpB*	antigen 85B	AADMWGPSSDPAWER	34580	5.62

[a]Predicted Mw and pI values calculated using the Expasy compute pI/Mw tool (http://www.expasy.ch/tools/pi_tool.html).
[b]Novel proteins not previously identified by proteomics (Jungblut *et al.* 1999; Rosenkrands *et al.* 2000a and b; http://www.mpiib-berlin.mpg.de/2D-PAGE).

4.3.2 Reactivity of monoclonal antibodies with proteins extracted from *M. tuberculosis* strains

In some cases, spots excised from 2D gels were identified as containing two or more proteins by LC/MS/MS. This included proteins of the Ag85 complex and PstS1. Western blot analysis was therefore employed to help determine whether these proteins were responsible for the differences observed and also to investigate the expression of 12 other antigens in the three strains. Western blot analysis of whole-cell lysate and culture filtrate proteins harvested from the different *M. tuberculosis* strains showed that varying levels of the proteins to which the mAbs react were expressed in the different *M. tuberculosis* strains. The differences in mAb reactivity between the strains are shown in Figure 4.2 and Figure 4.3.

1D and 2D Western blotting identified PstS1 as the protein responsible for the decreased expression of spot 12 (Figure 4.1 and Table 4.2) in the Beijing strain compared to the family 23 strain and H37Rv (Figure 4.2D and Figure 4.3B). 2D Western blotting showed the existence of more than one isoform of PstS1, which has been previously reported by Sonnenberg & Beslisle (Sonnenberg & Beslisle, 1997) and is probably due to post-translational modifications such as glycosylation, phosphorylation or acetylation. The absence of PstS1 in culture filtrates of the family 23 strain (Figure 4.3B) is probably due to decreased expression in culture filtrates compared to whole-cell lysates, as observed on 1D Western blots (Figure 4.2D). Several isoforms of α-crystallin were observed and were expressed more highly in the Beijing strain compared to the family 23 strain and H37Rv (Figure 4.2A and Figure 4.3A). Consistent with 2D results, expression of α-crystallin was increased in the whole-cell lysate compared to the culture filtrate. L-alanine dehydrogenase and ESAT6 (Figure 4.2B and I) showed decreased expression in

H37Rv compared to both clinical strains, while expression of ESAT6 was decreased in the Beijing strain compared to the family 23 strain. Expression of L-alanine dehydrogenase was restricted to whole-cell lysates, whereas ESAT6 showed increased expression in both fractions. Three proteins, Hsp65, the Ag85 complex and the 47 kDa protein were downregulated in both clinical strains relative to H37Rv (Figure 4.2C, F and H), while Hsp65 and the 47 kDa protein were also downregulated in the Beijing strain compared to the family 23 strain. Multiple bands of Hsp65, possibly different isoforms or degradation products, were observed in both whole-cell lysates and culture filtrates. Hsp65 observed in culture filtrates is probably due to bacterial lysis. The expression of the other proteins was approximately similar in all the strains analysed, although some quantitative differences between their expression levels in whole-cell lysates and culture filtrates were observed. For example, expression of KatG was increased in the culture filtrate of H37Rv compared to the clinical strains, but decreased in the whole-cell lysate of H37Rv compared to the clinical strains (Figure 4.2E). The mAb directed to KatG (IT42) reacted with two bands in both whole-cell lysates and culture filtrates, with the lower band corresponding to the correct molecular weight. Sonnenberg & Beslisle (Sonnenberg & Beslisle, 1997) have also reported that IT42 reacts with more than one protein. The upregulation of α-crystallin and L-alanine dehydrogenase, and the down regulation of Hsp65 and Ag85 in the clinical strains compared to H37Rv, was confirmed by ELISA (data not shown).

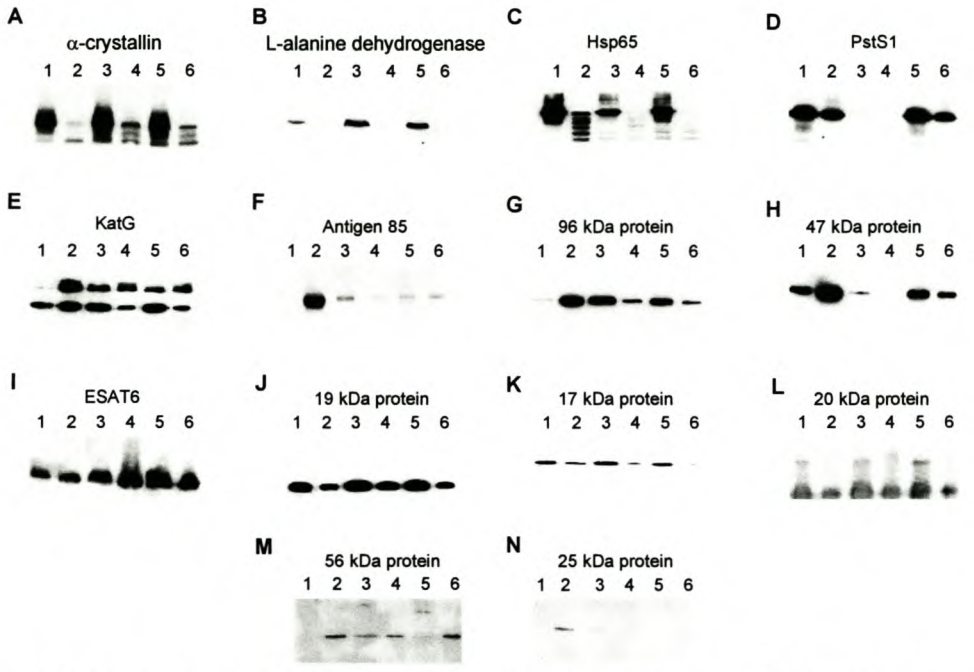

Figure 4.2 Protein expression in *M. tuberculosis* strains. Ten micrograms of whole-cell lysate (lanes 1, 3, 5) and culture filtrate proteins (lanes 2, 4, 6) of *M. tuberculosis* H37Rv (lanes 1, 2), the Beijing strain (lanes 3, 4) and the family 23 strain (lanes 5, 6) was separated by 12% SDS-PAGE, transferred onto PVDF membranes and probed with mAbs directed to α-crystallin (A), L-alanine dehydrogenase (B), Hsp65 (C), PstS1 (D), KatG (E), the Ag85 complex (F), the 96 kDa protein (G), the 47 kDa protein (H), ESAT6 (I), the 19 kDa protein (J), the 17 kDa protein (K), the 20 kDa protein (L), the 56 kDa protein (M) and the 25 kDa protein (N).

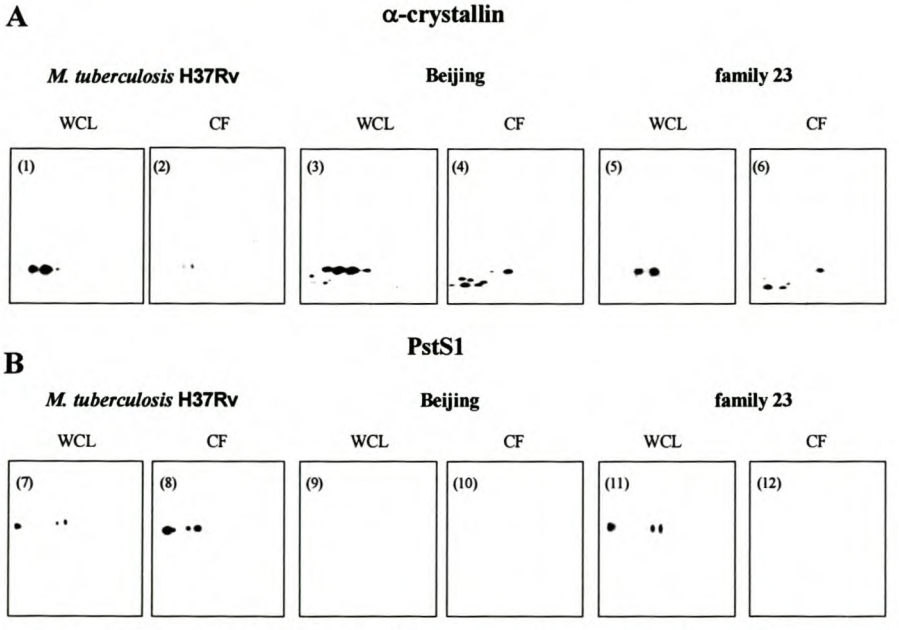

Figure 4.3 α-crystallin and PstS1 expression in *M. tuberculosis* strains. Sixty micrograms of protein extracted from *M. tuberculosis* H37Rv WCL (1, 7) and CF (2, 8), the Beijing strain WCL (3, 9) and CF (4, 10) and the family 23 strain WCL (5, 11) and CF (6, 12) was separated by 12% 2D PAGE, transferred to nitrocellulose membranes and probed with mAbs directed to α-crystallin (A) and PstS1 (B).

4.3.3 Reactivity of plasma samples to proteins extracted from the three *M. tuberculosis* strains

To establish whether there was differential antigen recognition in TB patients between the *M. tuberculosis* strains investigated in this study, plasma-derived antibodies of TB patients were tested against proteins extracted from *M. tuberculosis* H37Rv, the Beijing strain and the family 23 strain. After 1D PAGE separation and transfer to PVDF membranes, proteins were probed with plasma from TB patients infected with Beijing, family 23 or family 11 strains (Table 4.1, Figure 4.4). As expected, plasma from TB patients reacted with a wide variety of *M. tuberculosis* proteins, ranging from 10 kDa to over 105 kDa (Figure 4.4). Plasma samples were tested on antigens fractionated on different gels, therefore figures were aligned according to molecular weight markers, which were included on each gel, and mobility of immunodominant antigens. Our results clearly show that each patient had a characteristic banding pattern (Figure 4.4), with individual patients generally recognising the same proteins in *M. tuberculosis* H37Rv and the two clinical strains, as previously reported by Rojas-Espinosa *et al.* (Rojas-Espinosa *et al.* 1999).

Some differences in antigen recognition between the strains were noted. Prominent differences between strains are indicated by arrows in Figure 4.4. A band of approximately 56 kDa (arrow 1), was recognized by patient 880 in H37Rv but not in the family 23 strain or the Beijing strain (Figure 4.4F). Patient 880 reacted with a protein of about 35 kDa (arrow 2) in protein extracts of the family 23 strain and the Beijing strain only (Figure 4.4F). Proteins of approximately 28 and 25 kDa which were recognised strongly by patient 97 in H37Rv only, are indicated by arrows 3 and 4 (Figure 4.4A). Variation in antigen recognition was observed even between patients infected with the same strain (patients 941, 880 and 881 were infected with

Beijing strains, while patients 97, 484, 522 and 672 were infected with family 11 strains), suggesting that antibody responses are influenced by both *M. tuberculosis* strain type and patient-to-patient variation.

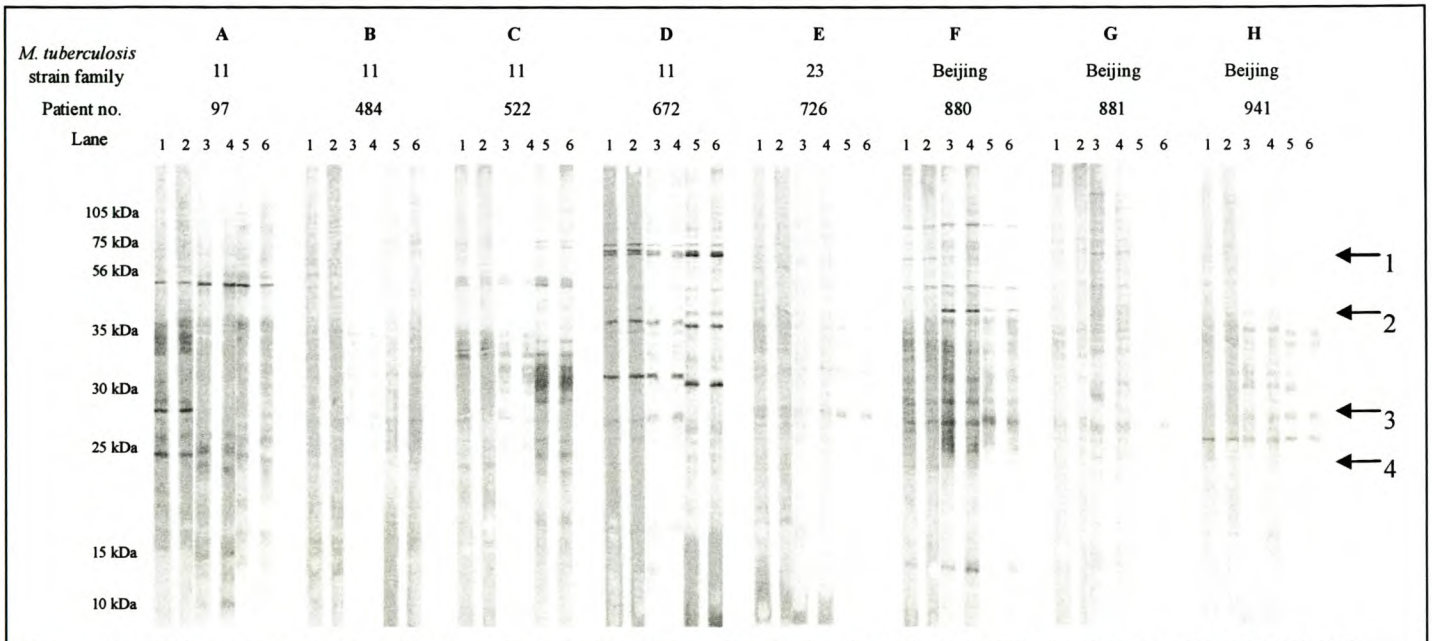

Figure 4.4 Antigen expression by *M. tuberculosis* strains. Plasma from TB patients (A) 97; (B) 484; (C) 522; (D) 672; (E) 726; (F) 880; (G) 881; (H) 941 was probed against proteins extracted from *M. tuberculosis* H37Rv (lane 1=1:100 plasma dilution; lane 2=1:500 plasma dilution), the Beijing strain (lane 3=1:100 plasma dilution; lane 4=1:500 plasma dilution) and the family 23 strain (lane 5=1:100 plasma dilution; lane 6=1:500 plasma dilution). Proteins were separated by 12% SDS-PAGE under reducing conditions and then transferred onto PVDF membranes. Molecular weight markers are indicated on the left. Arrows indicate antigenic variants at approximately 56, 35, 28 and 25 kDa.

2D Western blotting of patient plasma samples 97, 726, 880 and 941 against proteins extracted from *M. tuberculosis* H37Rv, the Beijing strain and the family 23 strain confirmed 1D Western blotting where each patient had a characteristic banding pattern (Figure 4.5). The changes observed in antigen recognition for patient 97 (Figure 4.4A) are obscured by a broad, diffuse smear on 2D blots (Figure 4.5). The smear may possibly represent lipoarabinomannan (LAM), an immunodominant glycolipid which migrates between 30 and 40 kDa, as demonstrated previously (Hunter *et al.* 1986). The 35 kDa and 56 kDa changes observed on 1D blots using patient 880 plasma (Figure 4.4F) are in a region containing many spots (Figure 4.5, box 4) on the 2D blots and therefore cannot be clearly identified. Patient 726 recognised more proteins in the Beijing strain compared to H37Rv and the family 23 strain (Figure 4.5, box 3). Boxes 1, 4 and 7 indicate regions where plasma from patients 97, 880 and 941 reacted with fewer proteins in the Beijing strain compared to the other strains. However these were not accurately resolved and may require larger format or narrow range IPGs for identification.

Patients 97 and 941 recognised two spots more strongly in the clinical strains than in H37Rv (Figure 4.5, spots 2 and 9), whereas patients 880 and 941 showed decreased recognition of spot 5 and spot 8 in the Beijing strain. Although it is difficult to speculate what proteins these spots represent, comparison with silver-stained 2D gels suggests that spot number 6, which is recognised by patient 880 only, represents α-crystallin. Interestingly, a band migrating at approximately the molecular weight of α-crystallin was also more prominently recognised by patient 880 on 1D blots (Figure 4.4F).

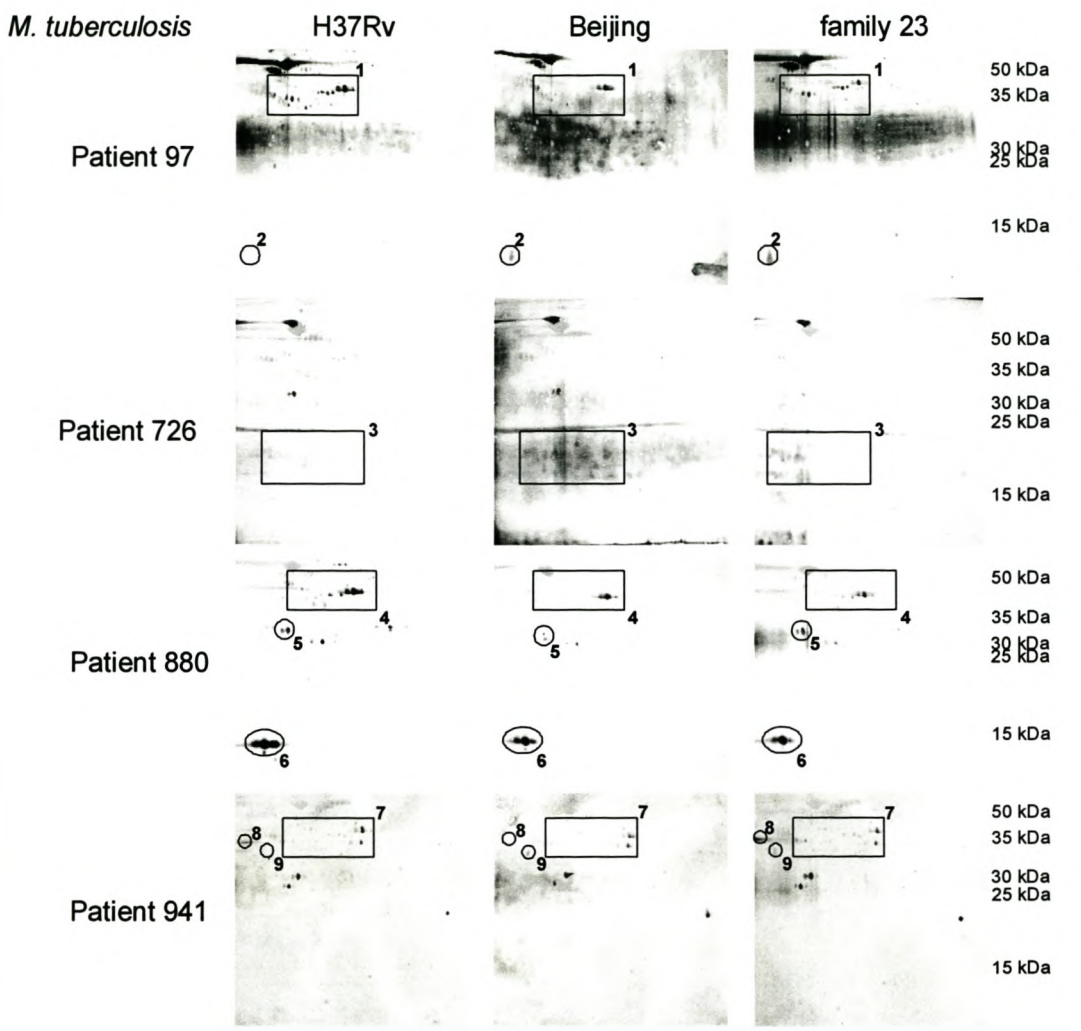

Figure 4.5 Antigen expression by *M. tuberculosis* strains. Proteins extracted from cultures of *M. tuberculosis* H37Rv, the Beijing strain and the family 23 strain were separated by 12% 2D PAGE, transferred to nitrocellulose membranes and probed with a 1:150 dilution of plasma samples from TB patients 97, 726, 880 and 941. Molecular weight markers are indicated on the right. Differential antigen recognition is indicated by circles and boxes.

4.3.4 Sequence comparisons of the promoter regions of the *katG* and *pstS1* genes of H37Rv, strain 1296 and the Beijing strain

Since there was a marked difference observed in the expression of PstS1 in the Beijing strain compared to the other strains and a difference in KatG expression between the clinical strains and H37Rv, the promoter regions of *pstS1* and *katG* in all three strains were analysed. Sequence comparison of the region encompassing 550 bp upstream and 150 bp downstream of the start codon of the *pstS1* and *katG* genes showed no difference between the published H37Rv sequence (http://genolist.pasteur.fr/TubercuList/) and sequences obtained from PCR products amplified from H37Rv, the Beijing and the family 23 strain (data not shown), suggesting that polymorphisms in this region are not responsible for the differential protein expression observed by these strains.

4.4 Discussion

Extensive research into development of serological tests to diagnose TB has failed to produce an accepted serodiagnostic test for clinical use (Chan *et al.* 2000). One of the factors hampering serodiagnosis is the humoral heterogeneity of TB patients (Lyashchenko *et al.* 1998a), which may be due to, amongst other things, differential antigen presentation by *M. tuberculosis* strains as they cause disease in different individuals (Lyashchenko *et al.* 1998a; Samanich *et al.* 2001). In this study we compared protein expression by *M. tuberculosis* H37Rv and two local clinical strains, one belonging to the Beijing family of strains and their recognition by the host humoral immune system. Several proteins showed increased or decreased expression in the clinical strains compared to the laboratory strain H37Rv. Additionally, the Beijing strain showed more marked increases or decreases in expression of some of these antigens compared to the other clinical strain. Furthermore, the probing of proteins extracted from the three strains by plasma from TB patients demonstrated differences in antigen recognition by these patients.

M. tuberculosis strains belonging to the Beijing family are distributed worldwide and are able to spread in large clonal clusters (Glynn *et al.* 2002). It is speculated that strains belonging to the Beijing family have an advantage over other strains of *M. tuberculosis* and that identification of the factors responsible would aid understanding of the success of Beijing strains compared to other *M. tuberculosis* strains (Bifani *et al.* 2002). It has previously been suggested that reduced expression of certain major antigens may allow strains to evade the host immune response (Stewart *et al.* 2001), and therefore the decreased expression of Hsp65, PstS1, ESAT6 and the 47 kDa protein by the Beijing strain compared to the family 23 strain as reported here, may contribute to the success of the Beijing strain family. Additionally, increased expression of factors such as α-crystallin which are induced under host-relevant conditions (Betts *et al.* 2002,

Monahan *et al.* 2001; Sherman *et al.* 2001; Yuan *et al.* 1998) may help to increase the pathogenicity and prevalence of Beijing strains compared to the family 23 strains. Differences in antigen recognition between the strains, as observed from 1D and 2D Western blots, may be significant and may assist the Beijing strain to minimise recognition by the host immune response, thereby facilitating the increased prevalence of Beijing strains.

Within our study community, Beijing strains have the highest mutation rate, due to IS*6110* insertions, chromosomal mutations and deletions (Warren *et al.* 2000). It has been shown that the IS*6110* element can insert into coding regions, thereby potentially affecting protein expression and phenotype (Sampson *et al.* 1999). Analysis of IS*6110* insertion sites in Beijing strains (Beggs *et al.* 2000; Sampson *et al.* 1999; Warren *et al.* 2000) showed that none of the genes investigated in this report were disrupted by IS*6110* insertion, suggesting that the differential protein expression observed in our Beijing strain compared to the other strains investigated is probably not IS*6110* insertion-mediated. However, the potential role of IS*6110* in affecting expression of these proteins cannot be discarded due to polarizing effects.

PstS1, a 38 kDa phosphate-binding lipoprotein possibly involved in phosphate transport, is specific for the *M. tuberculosis* complex, and is a known B-and T-cell stimulant (Harboe and Wiker, 1992). Although studies measuring antibody levels to PstS1 have reported that the presence of PstS1 antibodies is associated with advanced disease (Bothamley *et al.* 1992; Laal *et al.* 1997), PstS1 has shown the most promise for serological diagnosis of TB. Sensitivities of 70 % and 73 % have been reported for smear negative pulmonary TB and extrapulmonary TB patients, respectively (Wilkins and Ivanyi, 1990). In a recent evaluation of commercially available tests the highest sensitivity achieved with PstS1 was 55 % (Pottumarthy *et al.* 2000).

Decreased expression of PstS1 in the Beijing strain, as shown here, may possibly explain the absence of PstS1 antibodies in some TB patients. Similarly, differential expression of other proteins investigated in this study may contribute to the heterogeneous host humoral response of TB patients, suggesting that measurement of antibody levels to proteins that are not susceptible to differential expression by *M. tuberculosis* strains is required for the development of new diagnostic agents for TB. Furthermore, variation in antigen recognition by patients, as observed from Western blotting, suggests that both strain-related and host-related differences influence the humoral immune response, and may be responsible for the heterogeneity observed amongst TB patients (Lyashchenko *et al.* 1998a).

One dimensional Western blotting showed that smear-positive patients recognised more proteins than smear negative individuals. Studies have reported that higher bacterial loads *in vivo* result in increased antibody production (Chan *et al.* 2000). None of the patients used in this study were infected with HIV, dismissing an HIV-dependent reduced antibody response (Bothamley, 1995; Chan *et al.* 2000). 1D and 2D Western blotting showed that patient 97, who experienced two episodes of disease, recognised more antigens than any of the other patients. The increased antibody levels observed for patient 97 is possibly due to previous anti-TB therapy. Many studies have reported an increase in *M. tuberculosis* specific antibodies during treatment (Bothamley, 1995), possibly due to increased stimulation of the humoral response as antibiotic therapy destroys mycobacteria, and increases the antigenic load or, alternatively, due to the disappearance of circulating antigens and therefore release of antibodies trapped in immune complexes. Anti-TB treatment has been speculated to be one of the reasons for the heterogeneous humoral immune response of TB patients (Lyashchenko *et al.* 1998a). In this study we have observed variable antibody responses amongst patients who had not yet undergone drug treatment, suggesting that

although anti-TB treatment quantitatively affects antibody responses, it does not have a major effect on humoral heterogeneity.

This study has shown that both differential antigen presentation by *M. tuberculosis* strains and patient-to-patient variation affect the host humoral immune response, although patient-to-patient variation appears to be the major factor affecting humoral heterogeneity. Using 2D Western blots, Samanich *et al.* (2001) showed that both noncavitary and cavitary TB patients recognised a subset of 12-15 *M. tuberculosis* culture filtrate antigens, and reported that the humoral response of TB patients is homogeneous. Our conflicting results are possibly due to the fact that we measured antibody responses to whole-cell lysate proteins, whereas Samanich *et al.* (Samanich *et al.* 2001) looked at culture filtrate proteins. Furthermore, our patients were recruited from a high incidence community (Warren *et al.* 2000), where patients are infected with a wide variety of *M. tuberculosis* strains. Differential protein expression by *M. tuberculosis* strains, as shown in this study, may explain the high degree of antibody variability between our patients.

The finding that both host and bacteriological factors contribute to the humoral heterogeneity observed amongst TB patients may complicate the development of a serodiagnostic test for TB, suggesting that tests based on single antigens are likely to be of limited use and that multi-antigen cocktails are more likely to be successful (Al Zahrani *et al.* 2000; Amicosante *et al.* 1999). Our study supports the development of a diagnostic test based on detection of several *M. tuberculosis* antigens, thereby circumventing the heterogeneity of the host humoral immune response. Furthermore, we have shown that differential protein expression by *M. tuberculosis* strains may explain why certain strain families, such as the Beijing family, are more "successful" than others.

Chapter 5

INVESTIGATION OF PROTEIN AND ANTIGEN EXPRESSION BY *MYCOBACTERIUM TUBERCULOSIS* STRAINS UNDER STRESS CONDITIONS

The results presented in this chapter will be adapted and submitted as a manuscript, "Investigation of protein and antigen expression by *Mycobacterium tuberculosis* strains under stress conditions. Carmen Pheiffer, Joanna Betts, and Paul van Helden".

5.1 Introduction

Tuberculosis (TB) is a major source of morbidity and mortality worldwide, resulting in about 8 million new cases and 2 million deaths annually (Corbett *et al.* 2003). *M. tuberculosis* can cause both active disease and latent infection, where bacilli are in a nonreplicating or dormant state. Individuals with latent TB can remain asymptomatic for years, after which immunosuppression may result in reactivation of dormant bacilli and the establishment of clinical disease (Flynn and Chan, 2001). This is most clearly illustrated by HIV coinfection, which increases the 10% lifetime risk of TB activation to a 10% annual risk (Bloom and Murray, 1992). The increasing HIV pandemic, especially in areas where TB is endemic and where latent TB must be prevalent has created an urgent need to develop new antimycobacterial drugs (O'Brien and Nunn, 2001) and vaccines (Olsen and Andersen, 2003) to target latent TB and minimize the progression to active disease. Furthermore, the development of new drugs that will target latent TB may decrease the duration of TB treatment, thereby reducing patient relapse rates and minimizing the development of drug resistance.

It is thought that mycobacterial dormancy occurs within granulomas, probably due to oxygen and nutrient deprivation (Fenton and Vermeulen, 1996) and that survival of bacilli depends on their ability to mount an effective response to this hostile environment. A number of *in vitro* models to mimic the metabolic state of mycobacteria during latent disease have been developed and are used as tools for identifying proteins that are differentially expressed by dormant bacteria. The Wayne model mimics dormancy *in vitro* by subjecting bacterial cultures to a gradual depletion of oxygen (Wayne, 1994; Wayne and Hayes, 1996). During anaerobic conditions, bacteria terminate replication and adopt a persistent and viable state, capable of growth resumption with oxygen reintroduction. Bacteria in this hypoxic environment are resistant

to isoniazid and rifampicin, the frontline antimycobacterial drugs, and could possibly explain the persistence of the pathogen during chemotherapy (Wayne and Sramek, 1994). This model has been used extensively to identify mycobacterial factors expressed during oxygen depletion. A number of studies have shown upregulation of α-crystallin under conditions of hypoxia (Boon *et al.* 2001; Desjardin *et al.* 2001; Rosenkrands *et al.* 2002; Sherman *et al.* 2001; Tabira *et al.* 1998; Yuan *et al.* 1998). Other proteins including bacterioferritin and Rv2623 have also been reported to be induced during oxygen depletion (Boon *et al.* 2001; Sherman *et al.* 2001; Rosenkrands *et al.* 2002). Monahan *et al.* (2001) showed upregulation of α-crystallin and Rv2623 during *in vitro* macrophage infection, suggesting that these proteins may be important for intracellular survival. Further evidence for the role of α-crystallin in TB pathogenesis comes from studies reporting that α-crystallin is necessary for survival in cultured macrophages (Yuan *et al.* 1998), and is frequently recognized by sera from TB patients (Lee *et al.* 1992; Verbon *et al.* 1992).

Betts *et al.* (2002a) developed an *in vitro* model of persistence based on the results of Loebel *et al.* (1933). Here, transfer of cultures from a nutrient-rich medium to PBS arrests bacterial growth, decreases respiration, and alters antimycobacterial drug susceptibility (Betts *et al.* 2002a). Transcript expression profiling showed downregulation of genes involved in transcription and translation, energy metabolism, lipid biosynthesis, and cell division. Two genes involved in antibiotic resistance were upregulated and nutrient-starved bacteria were resistant to isoniazid, rifampicin and metronidazole. Resistance to metronidazole suggests that nutrient starvation does not induce hypoxia since bacilli are sensitive to metronidazole during anaerobic growth. Proteomics identified several proteins that were induced in response to nutrient starvation, and microarray analysis confirmed some of the changes at the gene level (Betts *et al.*

2002a). *In situ* hybridization showed that mRNA for Rv2557 and/or Rv2558, two genes upregulated during nutrient starvation (Betts *et al.* 2002a), is expressed in human lung granulomas and may therefore be important for the persistence of infection (Fenhalls *et al.* 2002). This study was not able to distinguish expression of Rv2557 and Rv2558 since these gene products are highly homologous (69% protein and 81% DNA identities).

In previous studies we compared protein expression and antigen presentation by two *M. tuberculosis* clinical strains, originally isolated from patients from a community in Cape Town, South Africa with a very high TB incidence (Warren *et al.* 2000), to the laboratory strain H37Rv using one-dimensional (1D) and two-dimensional (2D) polyacrylamide gel electrophoresis (PAGE), enzyme-linked immunosorbent assay (ELISA) and Western blotting (Pheiffer *et al.* 2002 and unpublished data). Based on the number of IS*6110* insertions and spoligotyping, the two clinical strains were classified as belonging to the Beijing family and family 23 (Pheiffer *et al.* 2002; Warren *et al.* 2000). Our results revealed considerable differences in protein expression between the three strains, particularly between the two clinical strains compared to H37Rv. Here, we have extended our previous studies by comparing protein expression and antigen recognition patterns of these strains during "dormancy" conditions. We chose to mimic dormancy using hypoxia (Wayne, 1994; Wayne and Hayes, 1996), nutrient depletion (Betts *et al.* 2002a), and stationary growth (Yuan *et al.* 1996). Protein expression during these conditions was compared to expression patterns obtained from log-phase cultures. Through 2D PAGE and silver staining, and Western blotting with monoclonal antibodies (mAbs) directed to well-known *M. tuberculosis* proteins, we were able to identify several proteins induced under these conditions. 2D Western blotting with patient sera identified several antigens which are differentially recognized by the host during "dormancy" conditions, thereby suggesting that at least some bacteria were likely in a

dormant or latent stage in the host. Comparison of the protein expression and antigen recognition profiles of *M. tuberculosis* H37Rv and two local clinical strains revealed some differences between the three strains. Careful analysis of these differences could identify factors important for latency, thereby aiding the development of new drugs and vaccines. Identification and analysis of differences observed between the clinical strains may provide some clues as to why patients do not develop adequate protective immunity to TB and why they are susceptible to reinfection. Furthermore, differences may also explain why some *M. tuberculosis* strain families are perceived to be more dominant than others.

5.2 Materials and Methods

5.2.1 *M. tuberculosis* strains and culture

M. tuberculosis H37Rv (ATCC 25618) and two local clinical strains (Beijing (SAWC 1524) and family 23 (SAWC 1296)) were grown in 10 ml of Middlebrook 7H9 media (Difco, Becton Dickinson, USA) supplemented with 10% albumin-dextrose-catalase (ADC) and 0.05% Tween-80 (MADCTW). Cultures were stirred with 8 mm Teflon-coated magnetic stirrer bars at 37°C until an optical density at 600 nm (OD_{600}) >0.6 was observed (LKB spectrophotometer, LKB Biochrom, Cambridge, UK). Pre-cultures were diluted 1:50 into 25 ml MADCTW and cultured for a further 7 days. For log-phase and stationary phase growth, pre-cultures were diluted 1:100 into 25 ml of MADCTW and stirred for 7 days (log-phase) or grown for 4 weeks without stirring (stationary) in 50 ml tubes (Corning, NY, USA). For nutrient starvation, exponential growing cultures were harvested, washed twice with phosphate buffered saline (PBS), resuspended in 25 ml of PBS and maintained at 37°C for 4 weeks without stirring in 50 ml tubes. For anaerobic conditions pre-cultures were diluted 1:10 into 50 ml MADCTW, and grown without stirring in 50 ml tubes. Tubes were sealed with parafilm. Oxygen depletion was monitored by addition of sterile methylene blue solution (500 µg/ml) to a final concentration of 1.5 µg/ml. Proteins were extracted after approximately 12 weeks when cultures appeared colourless. Morphology and acid-fastness was checked by Ziehl-Nielsen staining (Heifets and Good, 1994). All strains were sensitive to isoniazid, rifampicin and ethambutol.

5.2.2 Protein extraction

Whole-cell extracts were prepared as previously described (Pheiffer *et al.* 2002). Briefly, cultures were pelleted by centrifugation at 1 900 *g* for 20 min. Pellets were washed twice in

PBS/1% (v/v) Tween-20 and centrifuged (4 000 *g*, 5 min). Mycobacterial cells were resuspended in lysis buffer (0.3% (w/v) SDS, 200 mM DTT, 50 mM Tris-HCl pH 7.0, 1 mM PMSF and complete protease inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany)). An equal volume of Silica/Ceramic Matrix Beads (BIO 101, Vista, USA) was added, samples were mixed by vortexing and thereafter heat-killed at 80°C for 20 min prior to bead disruption in a ribolyser (speed of 6.5 for 45 seconds) (BIO 101, Vista, USA). After boiling for 5 min, samples were clarified by centrifugation at 10 000 *g* for 10 min and protein concentrations estimated using the Bradford assay (Bradford, 1976).

5.2.3 Two-dimensional polyacrylamide gel electrophoresis

Proteins were separated by two-dimensional gel electrophoresis as previously described (Betts *et al.* 2000), with minor modifications. Briefly, 20 µg (60 µg for Western blotting) of protein was resuspended in rehydration buffer (8 M urea, 2% (w/v) CHAPS, 10 mM DTT, 2% (v/v) immobilised pH gradient (IPG) buffer (pH4-7), trace bromophenol blue) and applied to pH 4-7 IPG strips (Amersham Biosciences, Uppsala, Sweden) for overnight rehydration. Isoelectric focusing was performed using a Multiphor II system (Amersham Biosciences) as follows: 100 V for 2 h, 300 V for 2 h, 1000 V for 1 h, 3500 V for 20 h (3500 V for 27.5 h for Western blotting). Second dimension separation was carried out by placing equilibrated IPG strips (Bjellqvist *et al.* 1993, http://www.expasy.ch/ch2d/protocols/) onto 12% PAGE gels and sealing with 0.5% (w/v) agarose in cathode buffer (defined below), containing a trace amount of bromophenol blue. Anode (375 mM Tris-HCl, pH 8.8) and cathode (192 M glycine, 0.1% (w/v) SDS, pH 8.3) buffers as described by Herbert *et al.* (1998) were used. Proteins were visualized by silver staining using an ammoniacal stain (Bjellqvist *et al.* 1993;

http://www.expasy.ch/ch2d/protocols/). Gels were air dried (BioRad GelAir Dryer, Hercules, USA) and compared by visualization on a light box.

5.2.4 Monoclonal antibodies

Mouse monoclonal antibodies (mAbs) IT-4, IT-13 and IT-23, raised against the 16 kDa (α-crystallin), 65 kDa (Hsp65) and 38 kDa (PstS1) *M. tuberculosis* proteins (Engers *et al.* 1986; Khanolkar *et al.* 1992; Sonnenberg and Beslisle, 1997) respectively were made available by the Department of Microbiology, Colorado State University through funds from the National Institutes of Health, National Institute of Allergy and Infectious Disease, Contract NO1-AI-75320. HYB76-8 directed to ESAT6 (Sorensen *et al.* 1995) was received from Karin Weldingh (Statens Serum Institut, Copenhagen, Denmark).

5.2.5 Western blotting with monoclonal antibodies

Ten micrograms of protein was separated by SDS-PAGE (Mini-PROTEIN electrophoresis unit, BioRad, Hercules, USA) using a 4% stack over a 12% resolving gel (Laemmli *et al.* 1970). After electrophoresis, proteins were transferred to PVDF membranes (Amersham Biosciences) by tank blotting. Blots were incubated with mAbs (IT-4, 1:1000; IT-13, 1:500; IT-23, 1:100; HYB76-8, 1:100) and screened for antibody binding with a horseradish peroxidase-conjugated goat anti-mouse IgG (1:10 000, CALTAG laboratories, Burlingame, USA). Bound antigens were detected using chemiluminescence detection reagents (ECL™ Western blotting detection reagents, Amersham Biosciences).

5.2.6 Western blotting with patient sera

2D gels were transferred to nitrocellulose membranes (S&S Protran BA85, Schleicher & Schuell Inc., Keene, USA) using semi-dry blotting. Blots obtained from 2D gels were probed with sera from a TB patient diluted 1:100 and screened for antibody binding using alkaline phosphatase-conjugated goat anti-human IgG (1:2 000, Kirkegaard & Perry Laboratories Inc., Gaithersburg, USA). Immunoreactive antigens were detected using the BCIP/NBT substrate (Kirkegaard & Perry Laboratories Inc.).

5.3 Results

5.3.1 Two-dimensional protein expression patterns of *M. tuberculosis* strains

Whole-cell lysate proteins were extracted from log-phase, nutrient-starved, stationary and anaerobic cultures of *M. tuberculosis* H37Rv, the Beijing strain and the family 23 strain. Proteins were analysed by 2D PAGE using pH 4-7 IPG strips and 12% PAGE gels (Figure 5.1). Pilot experiments demonstrated that many *M. tuberculosis* proteins focus between pH 4 and 7 and the best resolution for comparison was achieved using this pH range. Duplicate gels were run for each sample and proteins were visualized by silver staining. Spot differences obvious by visual comparison were analysed. Some of the proteins observed on 2D gels were putatively identified by comparison with previous 2D gels (Betts *et al.* 2002a; Pheiffer *et al.* unpublished data (chapter 4)) and their identities are listed in Table 5.3. Seven protein spots were downregulated in log-phase and nutrient-starved cultures of the Beijing strain compared to the *M. tuberculosis* H37Rv and the family 23 strain (Figure 5.1, Table 5.1, Table 5.3). Spots 3-6 were identified as α-crystallin, spot 12 contained Rv2629, while spots 1 and 2 each contained Rv2623. Spot number 1 also contained Rv2744c, the 35 kDa antigen. Previous studies have also reported the appearance of multiple isoforms of α-crystallin with different molecular weights and isoelectric points (Betts *et al.* 2000; Sonnenberg and Beslisle, 1997), possibly due to degradation, post-translational processing or post-translational modification, such as phosphorylation or glycosylation. Protein spot 13 was upregulated in log-phase and nutrient-starved cultures of the Beijing strain compared to H37Rv and the family 23 strain. Protein spot 11 was downregulated in proteins extracted from all cultures of the family 23 strain compared to *M. tuberculosis* H37Rv and the Beijing strain. Protein expression differences between H37Rv and both clinical strains were also noted. Protein spot 15, identified as Rv2624c, was downregulated in log-phase and nutrient-starved cultures of

the clinical strains compared to H37Rv. Spot 14 was only identified in log-phase and anaerobic cultures of the clinical strains compared to H37Rv. *M. tuberculosis* H37Rv, the Beijing strain and the family 23 strain showed similar expression of at least seven proteins, although varying levels of these proteins were expressed during the different culture conditions. Spot 18, Rv0685 or elongation factor EF-Tu, was downregulated in stationary and anaerobic cultures compared to log-phase and nutrient-starved cultures (Figure 5.1, Table 5.2, Table 5.3). Two spots (spot 7 and 8) identified as Rv2557 and Rv2558, were upregulated in nutrient-starved and stationary cultures compared to log-phase and anaerobic cultures. Spots 9 and 10 were downregulated in nutrient-starved cultures compared to log-phase cultures. Bacterioferritin, spot 16, was upregulated in stationary and anaerobic cultures compared to log-phase and nutrient-starved cultures. Spot 17 containing Rv2744c (the 36 kDa antigen) was upregulated in anaerobic cultures compared to log-phase, stationary-phase and nutrient-starved cultures. A number of the protein differences were not followed up due to limited resources. It was difficult to recognize some spot differences identified on 2D gels prepared from log-phase and nutrient-starved cultures on 2D gels prepared from stationary and anaerobic cultures, since protein spots were less defined on the latter gels.

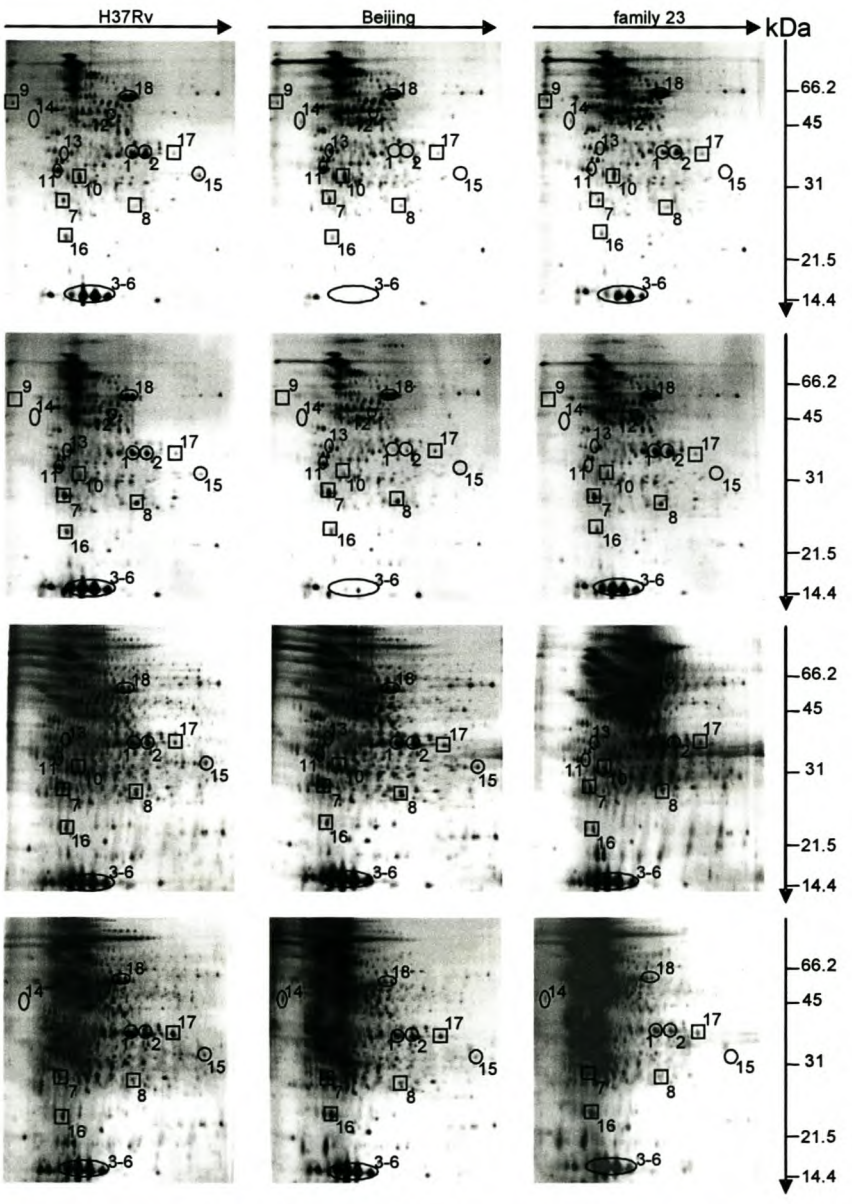

Figure 5.1 2D gels of proteins expressed during stress conditions. Proteins were extracted from log-phase, nutrient-starved, stationary and anaerobic cultures of *M. tuberculosis* H37Rv, the Beijing family strain and the family 23 strain. Protein spots with differential expression between strains and under culture conditions are circled, while proteins with similar expression between strains, but differential expression during culture conditions are boxed. The identities of some of the differences are listed in Table 5.3. Molecular weight markers are indicated on the right.

Table 5.1 Summary of protein spot differences between log-phase, nutrient-starved, stationary and anaerobic cultures of *M. tuberculosis* H37Rv, the Beijing strain and the family 23 strain.

	***M.tuberculosis* H37Rv**	**Beijing strain**	**Family 23 strain**
log-phase	standard*	Spots 1, 2, 3-6, 12 ↓	equals standard
		Spot 13 ↑	equals standard
		equals standard	Spot 11 ↓
		Spot 15 ↓	Spot 15 ↓
		Spot 14 ↑	Spot 14 ↑
nutrient-starved	standard*	Spots 1, 2, 3-6, 12 ↓	equals standard
		Spot 13 ↑	equals standard
		equals standard	Spot 11 ↓
		Spot 15 ↓	Spot 15 ↓
stationary	standard*	equals standard	Spot 11 ↓
anaerobic	standard*	Spot 14 ↑	Spot 14 ↑

*standard indicates the point of reference *M. tuberculosis* H37Rv.
The identities of some of the proteins are listed in Table 5.3.

Table 5.2 Protein spots with similar expression between strains but with varying levels of expression during growth conditions.

	log-phase	**nutrient-starved**	**stationary**	**anaerobic**
18	standard*	equals standard	↓	↓
7	standard*	↑	↑	equals standard
8	standard*	↑	↑	equals standard
9	standard*	↓	equals standard	equals standard
10	standard*	↓	equals standard	equals standard
16	standard*	equals standard	↑	↑
17	standard*	equals standard	equals standard	↑

*standard indicates the point of reference.
The identities of some of the proteins are listed in Table 5.3.

Table 5.3 Identity of some of the protein spots differences observed on 2D gels.

Spot	ORF	Gene name	Protein	Matched peptides
1	Rv2623	-	conserved hypothetical protein	YPNVAITR
				LLGSVSSGLLR
				SEEAQLVVVGSR
	Rv2744c	*35kd_ag*	35 kDa antigen	QALTLADQATAAGDAAK
2	Rv2623	-	conserved hypothetical protein	YPNVAITR
				LLGSVSSGLLR
				SEEAQLVVVGSR
3-6	Rv2031c	*hspX*	heat shock protein, HspX	SEFAYGSFVR
				TVSLPVGADEDDIK
7	Rv2557	-	24.3 kDa conserved hypothetical protein	Betts *et al.* 2002*
8	Rv2558	-	25.7 kDa conserved hypothetical protein	Betts *et al.* 2002*
12	Rv2629	-	hypothetical protein	TDLLSTLPQR
				IAPLDGVGALLR
				QELIDSLEEAVR
				DADMLSELGEPVDR
				GSGLAAQGLAEVCAALR
				LVDAADPEVVFVSGEVR
15	Rv2624c	-	conserved hypothetical protein	GPAGPVLVEASR
				DAEMICVGSVGIGR
				VQLAVIGGGEAGQLAR
				YASSILGSTATELAEK
				MTDAPDNEAVLEYAAR
16	Rv3841	*bfrB*	bacterioferritin	VEIPGVDTVR
				EALALALDQER
				HFYSQAVEER
				AGANLFELENFVAR
17	Rv2744c	*35kd_ag*	35 kDa antigen	LLSQLEQAK
				VQIQQAIEEAQR
				QALTLADQATAAGDAAK
				YANAIGSAELAESSVQGR
				SMSELAAPGNTPSLDEVR
18	Rv0685	*tuf*	elongation factor EF-Tu	TTVTGVEMFR
				LIQPVAMDEGLR
				AFDQIDNAPEER
				LLDQGQAGDNVGLLLR
				ELLAAQEFDEDAPVVR

Protein spots were putatively identified by comparison with previous 2D gels and mass spectrometry data (Pheiffer *et al.* unpublished data (chapter 4)).
*Spots 7 and 8 were identified by comparison with 2D gels and mass spectrometry data of Betts *et al.* 2002a.

5.3.2 Reactivity of monoclonal antibodies

Western blot analysis with monoclonal antibodies directed to ESAT6, Hsp65, PstS1 and α-crystallin was employed to investigate the expression of these proteins under the different growth conditions. Western blot analysis of whole-cell lysate proteins harvested from the different *M. tuberculosis* strains showed that expression of these proteins varied with growth condition and strain genotype (Figure 5.2). Expression of PstS1 appeared to be independent of growth condition, but dependent on strain genotype. PstS1 was not expressed by the Beijing strain compared to H37Rv and the family 23 strain. Levels of Hsp65 expression appeared to be maximal during log-phase, with no major differences in expression between strains. ESAT6 was expressed at lower levels by H37Rv compared to the clinical strains under all growth conditions studied. Expression of α-crystallin varied for the Beijing strain particularly, with no expression in log-phase cultures and nutrient-starved cultures. Expression of α-crystallin during stationary and anaerobic growth appeared to be higher for the Beijing strain compared to *M. tuberculosis* H37Rv and the family 23 strain. The lack of PstS1 expression by the Beijing strain, the increased expression of Hsp65 during exponential growth, the decreased expression of ESAT6 by H37Rv, and the absence of α-crystallin in log-phase and nutrient-starved cultures of the Beijing strain was confirmed by ELISA (data not shown).

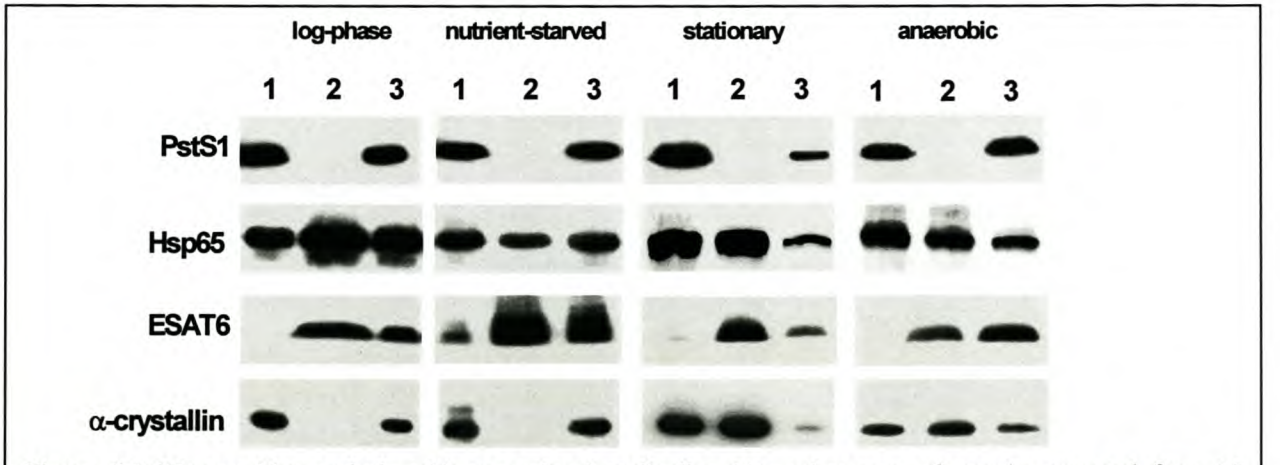

Figure 5.2 Western blot analysis with monoclonal antibodies. Ten microgram of protein extracted from *M. tuberculosis* H37Rv (lane 1), the Beijing strain (lane 2) and the family 23 strain (lane 3) was separated by 12% SDS-PAGE, transferred onto PVDF membranes and probed with mAbs directed to PstS1, Hsp65, ESAT6 and α-crystallin.

5.3.3 Antigen expression during different growth conditions

To determine whether the different growth conditions affected antigen recognition, serum from a TB patient was tested against proteins extracted from *M. tuberculosis* H37Rv, the Beijing and the family 23 strain. After 2D PAGE separation, proteins were transferred to nitrocellulose and probed with serum from a TB patient (Figure 5.3). Antibodies from the TB patient recognized a wide variety of antigens, which was dependent on culture condition, although also on strain type. Most of the antigens recognized were expressed during log-phase, nutrient-starvation and stationary growth, with only a few antigens recognized during anaerobic conditions. Antibodies from the TB patient recognized several proteins more strongly in anaerobic cultures, particularly for the clinical strains (Figure 5.3, circle 1). Proteins migrating in these positions on 2D gels of *M. tuberculosis* have previously been identified as different isoforms of α-crystallin (Betts *et al.* 2000; Jungblut *et al.* 1999). A number of studies have reported upregulation of α-crystallin during hypoxia (Boon *et al.* 2001; Desjardin *et al.* 2001; Rosenkrands *et al.* 2002; Sherman *et al.* 2001; Tabira *et al.* 1998; Yuan *et al.* 1998). Although some isoforms of α-crystallin were absent from silver-stained 2D gels of log-phase and nutrient-starved cultures of the Beijing strain, sera from the TB patient recognised α-crystallin in these cultures (Figure 5.3, circle 2). Western blotting with a mAb directed to α-crystallin confirmed the lack of α-crystallin in log-phase and nutrient-starved cultures of the Beijing strain. The discrepancy could be explained by the fact that 2D gels for Western blots contained three times the protein concentration used for silver-stained 2D gels and six times the protein concentration used for 1D Western blots. Patient sera recognized spot numbers 3 and 4 more strongly in log-phase cultures of clinical strains compared to nutrient-starved, stationary and anaerobic cultures (Figure 5.3). Antibodies from the TB patient did not recognize protein spots 5 and 6 in stationary

cultures of the family 23 strain, and in anaerobic cultures of both the Beijing and the family 23 strain (Figure 5.3). Generally, fewer antigens were recognised in stationary cultures of the family 23 strain compared to H37Rv and the Beijing strain. Spot 7 was detected only in stationary-phase cultures of the Beijing strain (Figure 5.3).

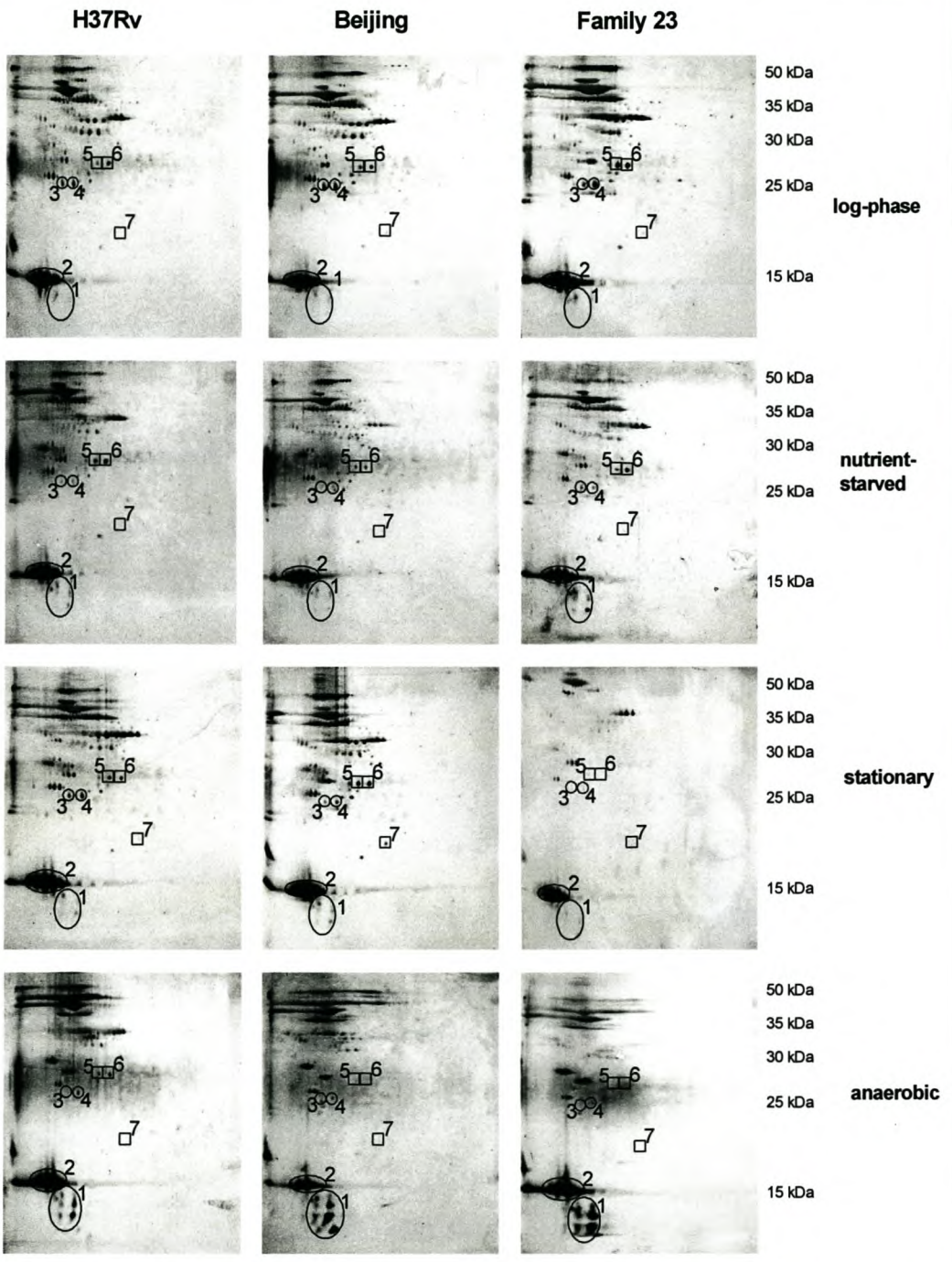

Figure 5.3 2D Western blot analysis with TB sera. Proteins extracted from log-phase, nutrient-starved, stationary and anaerobic cultures of *M. tuberculosis* H37Rv, the Beijing strain and the family 23 strain were separated by 12% 2D PAGE, transferred to nitrocellulose membranes and probed with TB sera diluted 1:100. Differential antigen recognition between strains under the different growth conditions is indicated by circles and boxes. Molecular weight markers are shown on the right.

5.4 Discussion

Identification of *M. tuberculosis* proteins expressed during dormancy could be important for the development of new antimycobacterial drugs and vaccines that will target latent infection. A number of studies have shown differential protein expression in *M. tuberculosis* H37Rv during dormancy (Betts *et al.* 2002a; Desjardin *et al.* 2001; Rosenkrands *et al.* 2002; Tabira *et al.* 1998; Yuan *et al.* 1998). Concerns that H37Rv may not be a valid model and the findings that virulence (Lopez *et al.* 2003) and protein expression patterns (Pheiffer *et al.* 2002) may vary for different *M. tuberculosis* strains, initiated the comparison of protein expression in H37Rv and two local clinical strains during dormancy conditions. The clinical strains investigated exhibited different frequencies in a TB high incidence community (Warren *et al.* 2000), and genotyping identified one of them as a member of the Beijing family. Expression patterns showed that some proteins were dependent on growth conditions, although some variation between strains was observed. Examination of the proteins extracted from each of these strains during the different growth conditions using sera from a TB patient showed differential antigen recognition under the different growth conditions and some differential antigen expression by the strains.

Various *in vitro* models to mimic mycobacterial dormancy have been developed, thereby aiding the identification of genes expressed during stress conditions. Using the Wayne model of oxygen depletion (Wayne, 1994; Wayne and Hayes, 1996) and the nutrient starvation model described by Betts *et al.* (2002a) we demonstrated that protein expression is dependent on both culture condition and on strain genotype. 2D gels showed that *M. tuberculosis* H37Rv and the family 23 strain expressed α-crystallin under all culture conditions, whereas the Beijing strain only expressed α-crystallin during stationary and anaerobic growth. The expression of α-

crystallin in the Beijing strain is consistent with studies showing upregulation during stationary growth (Yuan *et al.* 1996) and during hypoxia (Boon *et al.* 2001; Desjardin *et al.* 2001; Rosenkrands *et al.* 2002; Sherman *et al.* 2001; Tabira *et al.* 1998; Yuan *et al.* 1998). The trend of Rv2623 expression in the Beijing strain was similar to that of α-crystallin. Rv2623 has also been found to be upregulated during hypoxia (Boon *et al.* 2001; Sherman *et al.* 2001; Rosenkrands *et al.* 2002) and has recently been shown to lie within the same regulon as α-crystallin (Park *et al.* 2003). The expression of α-crystallin and Rv2623 in H37Rv and the family 23 strain were independent of culture condition. Differential expression of α-crystallin, a virulence factor (http://genolist.pasteur.fr/TubercuList/) during host-relevant conditions may help to increase the pathogenicity and prevalence of Beijing strains compared to the family 23 strains. PstS1 expression differed between the Beijing and the other two strains. PstS1 was undetectable in all cultures of the Beijing strain studied, but present in cultures from *M. tuberculosis* H37Rv and the family 23 strain. Previous studies have also suggested the absence of or low expression levels of PstS1 in the Beijing strain (Pheiffer *et al.* 2002 and unpublished data). Decreased expression of PstS1 in the Beijing strain, may possibly explain the absence of PstS1 antibodies in some TB patients, and why serodiagnosis of TB has been unsuccessful to date (Bothamley, 1995; Chan *et al.* 2000). In a similar vein, an unidentified protein appeared to be absent from family 23 strain compared to H37Rv and the Beijing strain during all growth conditions. Molecular epidemiology has shown that the Beijing strain is at least 10 fold more frequent than the family 23 strain (Warren *et al.* 2000). Our finding of differential protein expression by these strains may relate to strain frequency, as previous studies have reported that reduced expression of certain major antigens may allow strains to evade host immune responses (Stewart *et al.* 2001). Interestingly, probing proteins extracted from the *M. tuberculosis* strains with TB sera showed differential

antigen recognition, suggesting differential protein expression *in vivo*. Taken together, our results suggest that differential protein expression may explain why certain strain families are more dominant or "successful" than others, and why patients do not develop adequate protective immunity and are susceptible to reinfection.

The relevance of *M. tuberculosis* H37Rv as a model for human infection is crucial for the design of new vaccines and antimycobacterial drugs. H37Rv is the most commonly used laboratory strain and was isolated from a TB patient in 1905. Since then it has been continually passaged *in vitro* and *in vivo*, raising questions about its virulence and relevance as a model (Jacobs *et al.* 1996). Although H37Rv seems largely similar to clinical strains of *M. tuberculosis*, a few differences between H37Rv and the clinical strains were observed. Notably, expression of ESAT6 and a protein spot identified on 2D gels was decreased in H37Rv compared to the clinical strains. Although our results seem to support the use of H37Rv as a reference strain, we do not know how these differences may affect phenotype. 2D gels showed upregulation of Rv2557 and Rv2558 during nutrient-starvation and stationary growth in all the strains investigated. Upregulation of these proteins during nutrient starvation has previously been demonstrated in *M. tuberculosis* H37Rv (Betts *et al.* 2002a). We have confirmed the results of Betts *et al.* (2002a) and showed upregulation of Rv2557 and Rv2558 in two local clinical strains. These findings provide further evidence that H37Rv may indeed be a reasonable laboratory model.

Proteome examination using 2D gels showed only a few expression level differences between the strains under the culture conditions analysed. The failure to observe more differences could be due to several reasons including, 1) hydrophobic proteins are poorly soluble, 2) hydrophobic and high molecular weigh proteins will not enter the first dimension gels, 3) more

abundant proteins obscuring low copy proteins, 4) all *M. tuberculosis* proteins do not focus in the pH 4-7 range, 5) precipitation in the first dimension or 6) inefficient transfer of proteins to the 2D gel. Many studies have attempted to increase the resolution obtainable with 2D analysis. These include modification of sample preparation (Herbert, 1999), 2D analysis of subcellular fractions (Rosenkrands *et al.* 2000a) and using "proteomic contigs" (Urquhart *et al.* 1997). "Proteomic contigs" is the joining of protein maps formed by using a number of overlapping pH gradient strips to increase protein spot resolution. Future studies could investigate the response to other latency related factors such as low pH and reactive nitrogen intermediates. Furthermore, antigenic differences between strains and between the different growth conditions may be more pronounced in culture filtrates where the secreted proteins are found and future work should focus on these secreted proteins.

In conclusion, this study has shown that protein expression profiling and Western blotting may contribute to our understanding of the pathogenesis of tuberculosis by identifying specific *M. tuberculosis* gene products that are expressed in clinical strains during bacterial persistence. Careful analysis and identification of these differences may aid the development of new drugs and vaccines, and warrant further investigation. Rv2557 and/or Rv2558 seems like plausible chemotherapeutic targets for the persistent phase of infection (Fenhalls *et al.* 2002), although it is unlikely that these genes will make tractable drug targets since their function is not known and it would therefore be impossible to design a functional assay to identify inhibitors. These genes would possibly be potential vaccine candidates. The finding of differential protein expression by clinical strains may explain why certain strain families, such as the Beijing family, are more "successful" than others, and why patients are susceptible to reinfection. However, it is important

to correlate *in vitro* findings with latency models *in vivo*, therefore interesting findings should be validated in animal models.

Chapter 6

POTENTIAL FOR SERODIAGNOSIS OF TB IN A HIGH INCIDENCE COMMUNITY: MEASUREMENT OF CULTURE FILTRATE PROTEINS OF *MYCOBACTERIUM TUBERCULOSIS* (CFP), ANTIGEN 85 COMPLEX (AG85) AND LIPOARABINOMANNAN (LAM)-SPECIFIC IMMUNOGLOBULIN G (IgG)

The results presented in this chapter were presented at the **Fifth International Conference on the Pathogenesis of Mycobacterial Infections, Stockholm, Sweden, 2002,** as "**Antibody responses in patients from a high TB incidence area: effect of smear status, episode of disease and *M. tuberculosis* strain type**. Carmen Pheiffer, Joanna Betts, Pauline Lukey and Paul van Helden".

6.1 Introduction

A major obstacle in the eradication of TB is the lack of rapid and sensitive tests to diagnose TB early and thereby limit transmission and spread. Currently, TB is diagnosed by microscopic detection of acid-fast bacilli and/or bacteriological culture. However, these methods are not sensitive enough, detecting at best 60% of TB cases (Levy *et al.* 1989) and are relatively time-consuming. The development of systems utilizing radiometric and fluorescent-based culture systems have decreased the time required for culture (Pfyffer *et al.* 1997a, 1997b), but are thought to be too complex and expensive to use in developing countries where the greatest portion of the world's TB burden exists, and where they are most needed.

The need to develop a faster, easier, more sensitive and economical method to diagnose TB has sparked interest in serodiagnosis, especially in developing countries. Serology also has the potential to diagnose smear negative and extrapulmonary TB (Wilkins and Ivanyi, 1990), tuberculosis meningitis (TBM) (Chandramuki *et al.* 2002), and childhood TB (Gupta *et al.* 1997). Although many tests have been developed, no test has become accepted for general clinical use (Bothamley, 1995; Chan *et al.* 2000). The failure to develop a serodiagnostic test is thought to be due to the heterogeneous host humoral response, which is thought to be due to, amongst other things, host immunogenetics, extent of disease, bacillary load *in vivo*, antituberculosis therapy and differential gene expression by *M. tuberculosis* strains (Lyashchenko *et al.* 1998a).

The aim of this study was to assess the possibility of applying serology to diagnose TB in a high incidence setting in South Africa. South Africa has the third highest TB incidence worldwide, viz. 509/100 000 (Corbett *et al.* 2003). The community we investigated has

notification rates of TB above 1 000/100 000 (Beyers *et al.* 1996). We investigated whether bacterial load (smear status), disease episode and *M. tuberculosis* strain genotype (IS*6110* DNA fingerprint) affected antibody levels. Using enzyme-linked immunosorbent assay (ELISA) we measured *M. tuberculosis* culture filtrate proteins (CFP), antigen 85 (Ag85) and lipoarabinomannan (LAM)-specific immunoglobulin G (IgG). The IgG isotype has the most discriminative diagnostic potential (Daniel and Debanne, 1987; Kardjito *et al.* 1982). The antigens used in this study were previously identified to be strongly seroreactive (Laal *et al.* 1997; Samanich *et al.* 1998; Samanich *et al.* 2000). We show that serodiagnosis is potentially useful for the diagnosis of TB in a high incidence setting. Our results suggest that although bacillary load, disease episode and strain type may affect antibody levels, additional factors, such as host genetics are largely responsible for regulating humoral immunity.

6.2 Materials and Methods

6.2.1 Patients and controls

HIV negative individuals who were clinically diagnosed with TB and who were culture positive for drug sensitive *M. tuberculosis* strains were selected for this study. Samples were collected from TB patients at diagnosis. Patients were divided into two groups according to the presence (smear positive) or absence (smear negative) of acid-fast bacilli in sputum samples using the Ziehl-Nielsen stain. The genotyping of the infecting *M. tuberculosis* isolate was done using IS*6110*, as described elsewhere (Warren *et al.* 2000). Controls used in this study included, 1) apparently healthy individuals residing in the same community as the TB patients and with no previous diagnosis of TB (community controls), 2) Mantoux (PPD skin test) positive laboratory volunteers (Mantoux size of ≥15 mm) and 3) Mantoux negative laboratory volunteers (Mantoux size of <15 mm). Each group of five controls was pooled. Ethical approval for this study was obtained from the University of Stellenbosch Faculty of Health Sciences ethics committee and samples were only taken after informed consent was given.

6.2.2 Plasma samples

Blood was collected from patients and controls by venipuncture. Lymphocytes were allowed to settle at room temperature for approximately two hours and plasma was collected by removing supernatants. Plasma samples were clarified by centrifugation at 2 500 *g* for 30 seconds and was stored at -20°C until use.

6.2.3 *M. tuberculosis* antigens

M. tuberculosis antigens used in this study were obtained from Colorado State University. Proteins were purified from mid logarithmic phase *M. tuberculosis* H37Rv grown in GAS (Glycine Alanine salts) medium. Purified Protein Derivative (PPD), a mixture of extracellular proteins of *M. tuberculosis*, was supplied by the Ministry of Agriculture Fisheries and Food, Central Veterinary Laboratory, New Haw, Surrey.

6.2.4 *E. coli* whole-cell lysate proteins

Escherichia coli strains XL-1, DH5α, and top 10 were cultured with shaking at 37°C overnight. Cultures were centrifuged at 6 000 *g* for 15 min and pellets were resuspended in 0.1 M $NaHCO_3$/0.5 M NaCl pH 8.3. After pooling the three *E. coli* cultures, cells were sonicated for 2 min. Lysates were clarified by centrifugation at 10 000 *g* for 20 min and protein concentrations were determined using the Bradford assay (Bradford, 1976). One times (v/v) complete protease inhibitor cocktail (Roche Molecular Biochemicals) and 1 mM PMSF in isopropanol was added. Aliquots of *E. coli* proteins were stored at -70°C until use.

6.2.5 Enzyme-linked immunosorbent assay

Fifty microlitres of antigen diluted to 10 μg/ml (except for Ag85 and LAM, which were used at 1 μg/ml and 2 μg/ml, respectively) in 0.05 M $NaHCO_3$, was allowed to bind to duplicate wells of ELISA plates (Nunc, Roskilde, Denmark) at 4°C overnight. After three washes with PBS/0.05% (v/v) Tween-20 the wells were blocked with 2% (w/v) non-fat milk powder (Clover, Roodepoort, South Africa) in PBS/0.05% (v/v) Tween-20 (blocking buffer) for two hours at 37°C. Thereafter, fifty microlitres of plasma in blocking buffer (1:100-1:500) was added to the

wells and incubated for two hours at 37°C. Following this, plates were washed five times with PBS/0.05% (v/v) Tween-20 and 50 µl horseradish peroxidase (HRP)-conjugated goat anti-human immunoglobulin (IgG; CALTAG laboratories, USA) diluted 1:10 000 in blocking buffer was added to the wells and allowed to stand for one hour at 37°C. Plates were washed five times with PBS/0.05% (v/v) Tween-20 as before and developed with 100 µl of tetramethylbenzidine substrate (TMB, Kirkegaard & Perry Laboratories Inc. USA). After 10 minutes at room temperature, colour development was stopped by adding 100 µl 1 M H_2SO_4. Plates were read at 450 nm using a bench top microplate reader (Bio-Rad laboratories, USA). Three standard deviations (SD) above the optical density (OD) readings of controls were used as cut-off values to determine the sensitivity of the assays.

6.2.6 Standardization and statistical analysis

Assays were standardized for plate-to-plate and day-to-day variation by comparing the OD_{450} values of all test samples with a reference standard. Statistical analysis was done using GraphPad Prism version 2.01 (unpaired t tests).

6.3 Results

6.3.1 Patient data

The data of TB patients used in this study are shown in Table 6.1.

Table 6.1 Clinical details of subjects evaluated.

Patient	Smear status[a]	Disease episode[b]	Strain family[c]
1-4	-	1	0
5	+	1	2
6	-	1	2
7-8	-	1	11
9-14	+	1	11
15	+	1	14
16	-	1	14
17	-	1	21
18	+	1	23
19	+	1	28
20-26	+	1	29
27	+	2	4
28	-	2	9
29	-	2	11
30-31	+	2	11
32	+	2	14
33	+	3	10
34	-	3	14
35	-	4	11
36	+	4	29

[a]Smear status refers to whether acid-fast bacilli was detected (smear +) or not detected (smear -) in patient sputum samples using the Ziehl-Nielsen stain.
[b]Disease episode refers to the episode of disease the patient is currently experiencing (1-first episode, 2-second episode, etc.)
[c]Using IS*6110* genotyping, closely related *M. tuberculosis* strains were divided into families. In this study strains belonging to 11 different families were analysed (Warren *et al.* 2000).

6.3.2 Specificity of *M. tuberculosis* antigens

Individuals are likely to have antibodies to commensal bacteria, environmental bacteria and from BCG vaccination that may cross-react with many *M. tuberculosis* proteins (Laal *et al.* 1997). We compared the antibody response of 5 pooled TB patients and 5 pooled controls to *M. tuberculosis* CFP and whole-cell lysate (WCL) proteins, PPD and *E. coli* WCL proteins. Table 6.2 shows the ratio obtained by dividing OD_{450} readings of IgG responses of TB patients by the OD_{450} readings of IgG responses of controls. TB patients had significantly higher antibody responses than controls against the three *M. tuberculosis* antigens tested (range 3.9-8.34 fold higher). The ability to discriminate between TB patients and controls varied according to the *M. tuberculosis* antigen analysed. Depending on plasma dilutions used, the differences between TB patient and controls were between 3.9-6.2 fold, 5.46-8 fold, and 6.44-8.34 fold for WCL, CFP and PPD, respectively. The lowest discrimination between TB patients and controls was seen when measuring reactivity towards WCL proteins. This is expected, as the WCL contains many heat shock proteins that are conserved between most bacteria including mycobacteria (Shinnick *et al.* 1988). CFP and PPD contain mostly secreted proteins, most of which are *M. tuberculosis* specific (Andersen *et al.* 2000). No significant difference between TB patients and controls (1.33-1.61x) was seen when comparing antibody responses to *E. coli* WCL proteins. This is expected, as *E. coli* is a commensal pathogen and all individuals, healthy or diseased, will have *E. coli* reactive antibodies. Our results show that a higher plasma dilution improved the ability to discriminate between TB patients and controls, possibly due to an increase in antibody titres of patients during TB. The same antibodies may be present in controls but their titres will be lower than in active TB and therefore will not be detectable at higher dilutions.

Table 6.2 *M. tuberculosis* CFP and WCL, PPD and *E. coli* WCL-specific IgG levels of TB patients relative to controls. The IgG responses of TB patients obtained by ELISA, were divided by the IgG responses of controls (OD_{450} TB patients/OD_{450} controls), so that the numbers represent the ratio between TB patients and controls as explained.

	TB patient / control		
	100[a]	200[a]	300[a]
M. tuberculosis CFP	5.46	8	7.6
M. tuberculosis WCL	3.9	5.9	6.2
PPD	6.44	8.34	7.62
E. coli WCL	1.33	1.61	1.29

[a]Three different plasma dilutions were used, 1:100, 1:200 and 1:300.

6.3.3 Antibody responses of TB patients and controls against *M. tuberculosis* CFP, Ag85 and LAM

To minimize individual patient heterogeneity, plasma samples from TB patients and control individuals were pooled. IgG responses were measured in plasma pooled from 36 TB patients, 5 community controls, 5 Mantoux positive controls and 5 Mantoux negative laboratory controls with no evidence of active TB. The mean antibody levels of the various groups of subjects against CFP, Ag85 and LAM, using 1:100 (A) and 1:500 (B) plasma dilutions are shown in Figure 6.1. TB patients had higher antibody titres than controls against CFP, Ag85 and LAM. Mantoux positive laboratory staff generally had higher plasma antibody levels than Mantoux negative and community controls. This agrees with published results that showed raised antibody levels in PPD positive individuals (Kardjito *et al.* 1982). Community controls showed slightly higher antibody levels than Mantoux negative individuals at a dilution of 1:100, but similar levels were observed when analysing plasma at 1:500.

Different antigens were immunodominant in the groups of subjects investigated. TB patients and Mantoux positive laboratory controls recognized LAM most strongly, followed by Ag85 and CFP. Mantoux negative laboratory controls and community controls recognized Ag85 most strongly, followed by LAM and CFP. Our results agree with previous findings from Bothamley *et al.* (1992b), who also showed differences in antigen recognition in exposed versus non-exposed individuals.

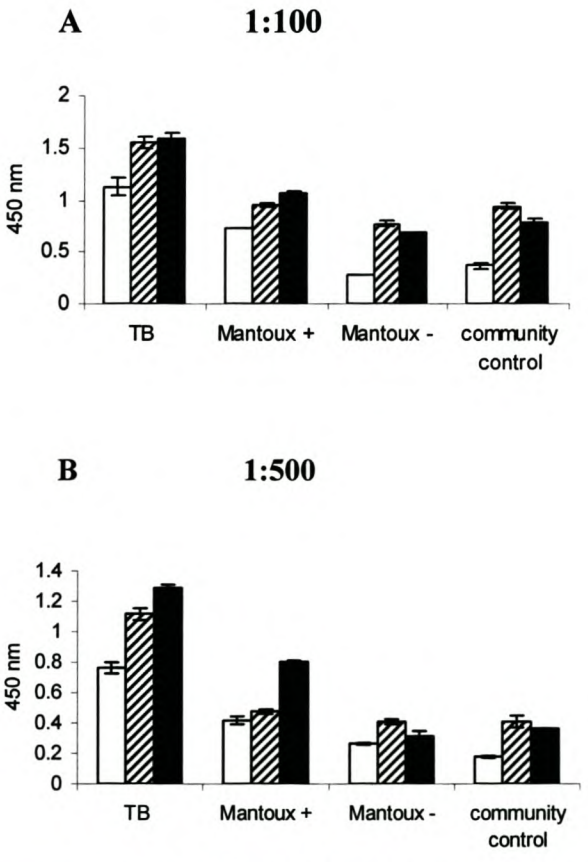

Figure 6.1 Measurement of IgG levels to CFP, Ag85 and LAM. IgG levels of TB patients, Mantoux positive and Mantoux negative volunteers and community controls to culture filtrate proteins (CFP) (clear bars), antigen 85 (Ag85) (striped bars), and lipoarabinomannan (LAM) (shaded bars) was measured using plasma diluted 1:100 (A) and 1:500 (B). Error bars represent standard deviation between duplicate samples.

6.3.4 Reactivity of TB patients

Antibody responses of 36 individual TB patients were characterized by measuring the response of each patient to CFP, Ag85 and LAM. Sensitivity refers to the ability of a test to correctly identify TB positive individuals. The mean OD_{450} readings plus three standard deviations (SD) obtained with Mantoux positive individuals, Mantoux negative individuals or community controls were used as cut-off values to allow discrimination between TB patients and controls. The sensitivity of the assay varied with cut-off values chosen, antigen tested and with plasma dilution used (Table 6.3). Sensitivities when using Mantoux negative controls as a cut-off were 66.7-80.6%, 69.4-75% and 77.7% for CFP, Ag85 and LAM, respectively. Sensitivities ranged between 44.4-63.8% and 58.3-83.3% for Mantoux positive and community controls, respectively. The overall sensitivity when combining all three antigens was 94.4% (100% for smear positive, 85.7% for smear negative), 77.8% (84.2% for smear positive, 71.4% for smear negative), and 88.8% (94.7% for smear positive, 78.6% for smear negative) using cut-off values equal to the response of Mantoux negative, Mantoux positive and community controls, respectively.

Table 6.3 Sensitivity of assay using different groups of controls as cut-off values. The percentage of TB patients reactive to CFP, Ag85 and LAM was measured using OD_{450} readings of Mantoux positive, Mantoux negative and community controls plus 3 standard deviations (SD) of the mean as cut-off values.

	CFP		Ag85		LAM	
	1:100[a]	1:500[a]	1:100[a]	1:500[a]	1:100[a]	1:500[a]
[b]Mantoux positive	44.4%	44.4%	58.3%	63.8%	52.8%	50%
[c]Mantoux negative	80.6%	66.7%	69.4%	75%	77.7%	77.7%
[d]Community control	72.2%	83.3%	58.3%	63.9%	75%	80.6%

[a]Plasma samples were diluted 1:100 and 1:500.

[b]Mantoux positive and [c]Mantoux negative controls refers to PPD skin test positive and PPD skin test negative laboratory individuals, respectively.

[d]Community controls are controls recruited from the same community as TB patients.

We compared antibody levels to CFP, Ag85 and LAM with patient smear status (Figure 6.2) to determine whether bacterial load *in vivo* affects antibody levels as previously suggested by Lyashchenko *et al.* (1998a). Patients were defined as smear positive when acid-fast bacilli were detected in sputum samples by the Ziehl-Nielsen stain, or smear negative when no bacilli was detected. Our results show that the mean antibody responses of smear positive patients were slightly higher than that of smear negative patients for CFP (p=0.1105), Ag85 (p=0.9307) and LAM (p=0.6324), although differences were non-significant.

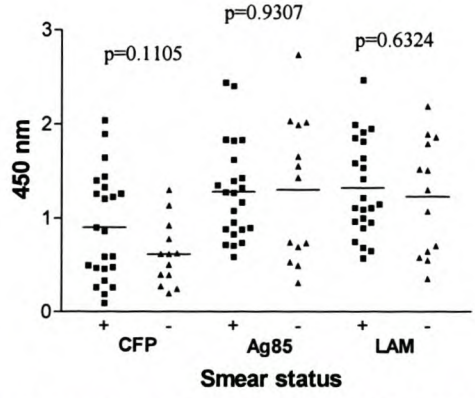

Figure 6.2 IgG levels and smear status. *M. tuberculosis* CFP, Ag85 and LAM-specific IgG levels in sputum smear positive (+, ■) and smear negative (-, ▲) patients. Mean antibody levels are indicated for each group of patients.

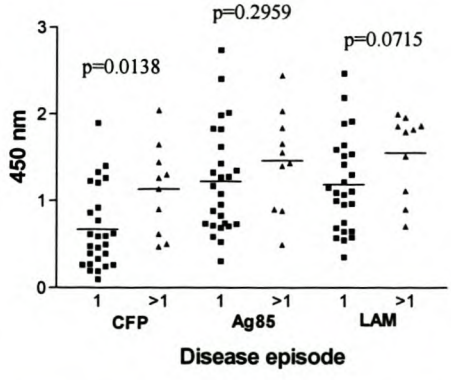

Figure 6.3 IgG levels and disease episode. *M. tuberculosis* CFP, Ag85 and LAM-specific IgG levels in patients experiencing a primary episode of TB (1, ■) and patients presenting with repeat episodes of TB (>1, ▲). Mean antibody levels are indicated for each group of patients.

Antibody titres of TB patients increase during antituberculosis chemotherapy, suggesting that patients with repeat episodes of TB may have higher antibody titres than patients with primary TB (Bothamley and Rudd, 1994; Imaz and Zerbini, 2000). We compared patient disease episode with antibody levels to CFP, Ag85 and LAM (Figure 6.3). Repeat episode patients had higher antibody levels than patients presenting with a first TB episode to CFP ($p=0.0138$), Ag85 ($p=0.2959$) and LAM ($p=0.0715$), with the only significant difference at 95% confidence level between the two groups observed for CFP-specific IgG, but at 92% level also for LAM.

It has been suggested that bacteriological factors may affect the humoral response (Lyashchenko *et al.* 1998a). We investigated whether *M. tuberculosis* CFP, Ag85 and LAM-specific IgG correlated with *M. tuberculosis* strain, as genotyped using IS*6110*. Figure 6.4 shows a comparison between *M. tuberculosis* strain type and IgG levels to CFP, Ag85 and LAM. Although most strain families are not well represented in our cohort, comparing antibody responses of patients infected with strain families 11 and 29 showed that patients infected with family 11 strains generally had higher antibody levels, although the differences were not significant.

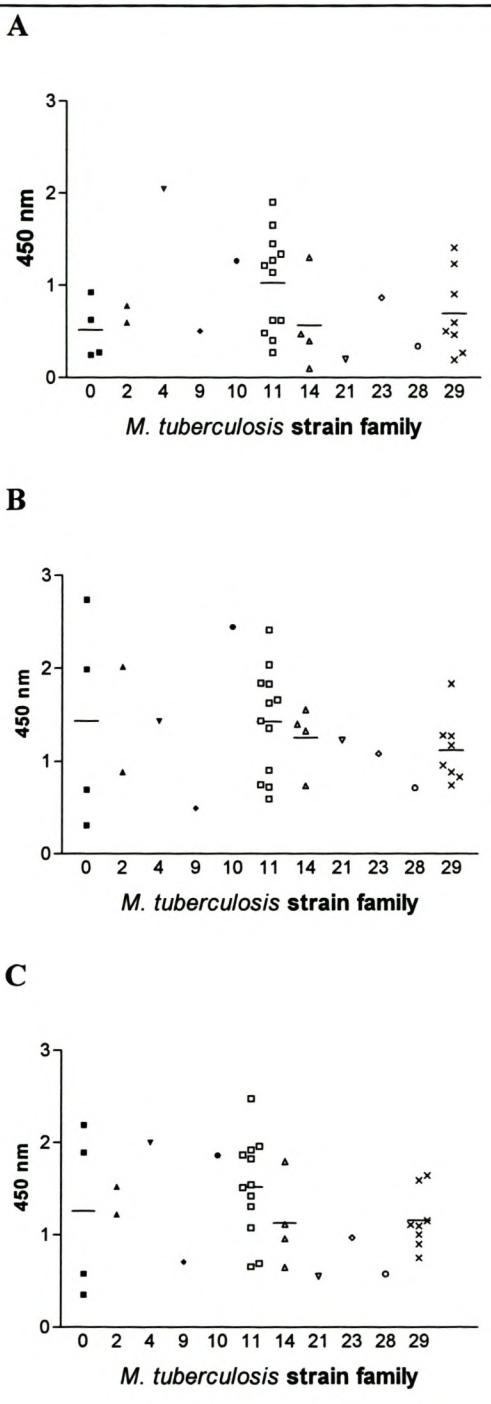

Figure 6.4 IgG levels and IS*6110* genotype. Antibody responses of patients against CFP (A), Ag85 (B) and LAM (C) were correlated with the genotype of their infecting *M. tuberculosis* isolate to determine if there is a correlation between antibody responses and strain type. The mean antibody response is indicated where more than three patients were infected with the same strain type.

6.4 Discussion

One of the major problems facing TB control is the lack of inexpensive, rapid, easy and accurate diagnosis of TB, particularly in developing countries. Serodiagnosis offers several advantages over traditional methods and is potentially more amenable for use in developing countries than other more recently developed methods (Bothamley, 1995; Chan *et al.* 2000; Daniel and Debanne, 1987). However, one of the major factors hampering the use of serology to diagnose TB is the heterogeneous humoral immune response of TB patients (Lyashchenko *et al.* 1998a). In this study we wanted to determine whether serodiagnosis could potentially be applied in a high TB incidence area. Furthermore, we investigated factors that could possibly explain the heterogeneity in antibody levels of TB patients.

The success of a serodiagnostic test is dependent on the cut-off values chosen (Daniel and Debanne, 1987). We decided to assess the potential of serodiagnosis in our community using three different control groups as cut-off, unexposed individuals (Mantoux negative laboratory volunteers) and exposed individuals (Mantoux positive laboratory volunteers and controls recruited from the same community as the TB patients (community controls)). There was no significant difference in the sensitivity of the assay when using Mantoux negative laboratory volunteers or community controls as cut-offs, although the sensitivity of the test was significantly reduced when using Mantoux positive laboratory volunteers. The reasons for the low antibody titres in community controls are not known since these individuals are thought to be constantly exposed to TB. A possible explanation is that strong cell-mediated immune responses, and therefore low antibody responses, in these individuals protect them against development of active TB (Bhatnager *et al.* 1977; Daniel *et al.* 1981; Lenzini *et al.* 1977).

Sensitivities obtained using single antigen preparations were not ideal. Combining reactivities to all three antigens showed at least 84.2% and 71.4% sensitivity in smear positive and smear negative patients, respectively, irrespective of the control group used as cut-off. These results support the idea that the development of serodiagnostic tests should be based on multi-antigen cocktails (Al Zahrani *et al.* 2000; Amicosante *et al.* 1999). These results are promising, especially since at least 50% of pulmonary TB patients are smear negative when reporting to clinics for the first time (Corbett *et al.* 2003) and culture will confirm TB in only about 50% of these individuals (Levy *et al.* 1989). Although smear negative TB is arguably less infectious and lower case mortalities are observed with smear negative patients, more than 50% of smear negative cases progress to active disease and transmission still occurs, albeit at a reduced rate (Behr *et al.* 1999a; Coleblunders *et al.* 2000).

Various factors, including bacterial load *in vivo* (Samanich *et al.* 1998), antituberculosis treatment (Bothamley and Rudd, 1994; Imaz and Zerbini, 2000), and strain type (Lyashchenko *et al.* 1998a; Samanich *et al.* 2001) may affect antibody responses. We compared antibody responses between smear positive and smear negative patients, patients presenting with their first disease episode and repeat episode TB patients, and patients infected with genotypically different *M. tuberculosis* strains. Our results show that although generally non-significant, smear positive individuals had higher antibody levels than smear negative patients. Increased bacterial load, as observed in smear positive compared to smear negative individuals, decreases cell-mediated responses (Spellberg and Edwards, 2001), and therefore higher antibody levels are expected in smear positive individuals (Bhatnager *et al.* 1977; Daniel *et al.* 1981; Lenzini *et al.* 1977). Patients with recurrent TB tended to have increased antibody titres, compared to patients with

newly acquired TB. This finding is consistent with studies reporting that antibody titres increase during the course of antituberculosis therapy and persist for up to 3 years after treatment (Bothamley and Rudd, 1994; Imaz and Zerbini, 2000). Our results suggest that the time since the previous TB episode should be taken into account for recurrent patients, to avoid false positivity due to antibody persistence after previous treatment. Ideally, antibodies that are not affected by antituberculosis treatment or antibodies that are involved in disease progression should be used for a serodiagnostic test, enabling discrimination between active and cured TB patients.

Correlating patient CFP, Ag85 and LAM-specific IgG levels with the genotype of the *M. tuberculosis* strain isolated from the patient sputum (Warren *et al.* 2000) showed no significant correlation between antibody levels and strain genotype. Molecular epidemiological studies in our community have shown that strain families 11 and 29 are responsible for 24.5% and 18.7% of TB cases in our community respectively (Warren *et al.* 2000). Our results show that patients infected with family 11 strains have higher antibody levels than patients infected with strain 29 families, irrespective of their bacillary load or disease episode. The hypothesis that antibody responses are inversely proportional to cell-mediated immune response may explain why family 11 strains are more frequent than family 29 strains, as patients infected by these strains may develop weaker cell-mediated responses, which are thought to be protective against TB (Bhatnager *et al.* 1977; Daniel *et al.* 1981; Lenzini *et al.* 1977). This hypothesis could potentially be confirmed by analysing the chest x-rays of these patients to determine whether antibody responses correlate with increased tissue damage and cavity formation.

Our results support earlier studies that report the heterogeneous antibody response of TB patients (Lyashchenko *et al.* 1998a), but in addition demonstrate the effect of bacterial load *in*

vivo, disease episode and *M. tuberculosis* strain genotype on antibody levels. Our findings seem to suggest that the above-mentioned factors minimally affect antibody levels, and we feel that additional factors, such as the extent of disease (Samanich *et al.* 1998) and the immunogenetic background of the host (Bothamley *et al.* 1989) are the major contributory factors. We have recently shown that antigen expression in *M. tuberculosis* varies with growth stage and to a lesser extent with strain type (Pheiffer *et al.* 2002). Lyashchenko *et al.* (1998b) demonstrated that different antigens become immunodominant over time during experimental bovine TB, further supporting the hypothesis that the heterogeneous host humoral immune response may be due to differential gene expression by *M. tuberculosis* strains during the course of disease. Furthermore, Lyashchenko *et al.* (1998b) showed that cattle infected with the same strain of *M. bovis* also develop a heterogeneous humoral response, showing that strain type alone does not dictate the humoral response and supporting the involvement of host factors in antibody production. Our findings suggest that regulation of the humoral response is complex and that a number of factors work together to affect humoral immunity.

In conclusion, our results suggest that serodiagnosis of TB patients, especially smear negative cases, in a high incidence setting such as ours may be possible and provides justification for further studies in larger cohorts. Our results show promise and development of the type of analysis used in this study could greatly assist earlier case detection with prompt instigation of treatment and therefore limitation of disease transmission, the main cause of recurrent TB after successful cure in our community (van Rie *et al.* 1999a). Future studies should assess the efficacy of assays, such as the one described here, in TB patients dually infected with human immunodeficiency virus (HIV). The increasing prevalence of HIV infection, especially in areas where TB is epidemic (Corbett *et al.* 2003) has provided further support for the development of

serodiagnostic tests, however there are conflicting reports in the literature about whether the immunosuppression observed after HIV infection affects the humoral response, which could potentially hamper serodiagnosis of TB (Wilkins, 1994).

Chapter 7

ANTIBODY RESPONSES OF PATIENTS DURING ANTIMYCOBACTERIAL TREATMENT

7.1 INTRODUCTION

Antimycobacterial chemotherapy to treat tuberculosis (TB) involves taking a combination of drugs for at least six months because the current drugs available are targeted against actively replicating bacteria and are less active against "persistent" bacilli (Parrish *et al.* 1998). The development of new drugs that will eradicate "persistent" bacilli, thereby shortening the length of treatment, reduce the risk of patient relapse, and minimize the development of multiple drug resistance is urgently needed (O'Brien and Nunn, 2001). These drugs should probably also be able to eliminate latent TB, a source for great concern with the HIV/AIDS epidemic (Corbett *et al.* 2003). However, the development of new drugs is hindered by numerous problems, such as the lengthy duration of clinical trials and associated high costs.

In an attempt to speed up the clinical evaluation of new drugs, various researchers are exploring the possibility of identifying surrogate markers of disease eradication. Surrogate markers could be either bacterial or host factors which are expressed early during treatment and which should predict patient outcome. Mitchison (1993) reported that patient sputum culture conversion after two months of antimycobacterial chemotherapy is one possible predictor of treatment success or failure. However, the limited predictive value and the relatively long culture period required for identification of *M. tuberculosis* is a major limitation and makes culture unsuitable as a surrogate marker. Monitoring sputum smears of refugees in Thailand has shown that sputum smear conversion after two months of DOTS appears to correlate with clinical outcome (Rieder, 1996), and is currently the most widely used method to monitor the efficacy of antimycobacterial chemotherapy. This method, while simple and inexpensive, is not as accurate as culture testing and relatively insensitive (Levy *et al.* 1989). Other potential surrogate markers, such as detection of mycobacterial growth in mycobacterial growth indicator tubes (MGIT)

(Epstein *et al.* 1998), quantifying *M. tuberculosis* antigen (Wallis *et al.* 1998), *M. tuberculosis* messenger RNA (mRNA) (Desjardin *et al.* 1999; Hellyer *et al.* 1999) and host cytokine levels (Ribeiro-Rodrigues *et al.* 2002) in patient sputum, and measuring antibody secreting cells (Sousa *et al.* 2000), interferon (IFN) γ producing T cells (Ulrichs *et al.* 2000), and immunoglobulin (Ig) G levels (Drowart *et al.* 1991; Hwang and Kim, 1993; Sánchez-Rodriguez *et al.* 2002; Sousa *et al.* 2000) in patient blood have also been investigated.

Serum antibody levels may reflect disease progression and may give important clues regarding treatment outcome. Antibody titres increase during the first weeks of treatment, possibly due to rapid bacterial killing and antigen release, and/or the disappearance of circulating immune complexes (Bothamley, 1995). Antibodies of different specificity are expressed during treatment, suggesting that the antigenic make-up of *M. tuberculosis* evolves in relation to disease progression (Bothamley *et al.* 1992a; Imaz and Zerbini, 2000). Using antibodies as surrogate markers to predict treatment outcome offers several advantages over other methods, which include accessibility of blood, speed of antibody detection assays, it obviates the requirement of cell culture and minimizes the risk of infection to laboratory workers.

In this study we report the measurement of Antigen 85B (Ag85B) (the dominant member of the Ag85 complex (Harth *et al.* 1996)) specific-IgG1, IgG2, IgG3 and IgG4 by enzyme-linked immunosorbent assay (ELISA). The aim of this work was to determine whether IgG subtype levels can differentiate between treatment "responders" (defined as those who sputum smear converted after two months of therapy) and "nonresponders" (defined as those who remained smear positive after two months of therapy) after four weeks of antimycobacterial chemotherapy.

Measurement of IgG subclasses, which are distinct in terms of structure, function and levels in serum (Shakib and Stanworth, 1980), is possibly more informative than measurement of total IgG (Gibson *et al.* 1987; Hussain *et al.* 1995, 2000, 2001). We chose to measure anti-Ag85B antibody levels since isoniazid, one of the frontline antituberculosis drugs, induces Ag85 expression (Garbe *et al.* 1996), and Wallis *et al.* (1998) reported that monitoring Ag85 levels in sputum may be useful to evaluate treatment outcome.

7.2 MATERIALS AND METHODS

7.2.1 Patients

Patients with primary pulmonary TB (sputum smear positive) were recruited from a suburb in the Western Cape Region of South Africa, with notification rates of TB above 1 000/100 000 (Beyers *et al.* 1996). Patients were diagnosed by microscopic detection of acid-fast bacilli in their sputum, bacteriological culture and radiographic examination. All patients were HIV negative, culture positive for drug sensitive *M. tuberculosis* and did not have any other diseases known to affect the immune system at the time of diagnosis. Patients received combination therapy under supervision (DOTS) as prescribed by the South African National TB Programme, which is based on WHO guidelines. The intensive phase (two months) consisted of daily doses of isoniazid, rifampicin, pyrazinamide and ethambutol, while the continuation phase (four months) consisted of daily doses of only isoniazid and rifampicin. All patients were fully compliant with treatment. Compliance is defined as taking at least 80% of the prescribed drug regimen during antimycobacterial chemotherapy. Patients were classified into two groups, "responders" and "nonresponders" according to their smear status after two months of antimycobacterial chemotherapy. "Responders" were defined as patients who responded to treatment and who smear converted after two months of treatment, while "nonresponders" were defined as patients who remained smear positive after two months of treatment. Ethical approval for this study was obtained from the Ethics committee, Faculty of Health Sciences, University of Stellenbosch. Permission for this work was also given by the Head of Health Services in the City of Tygerberg.

7.2.2 Sera

After informed consent, blood was collected by venipuncture at diagnosis and after four weeks of treatment. Blood was allowed to coagulate at room temperature for one hour and serum was collected by centrifugation at 2 500 g for 7 minutes at 4°C, aliquoted into 0.5 ml and stored at -70°C until use.

7.2.3 Antigens

Recombinant *M. tuberculosis* Ag85B expressed in *Escherichia coli* was purchased from Lionex Diagnostics and Therapeutics, Braunschweig, Germany. Purified IgG1, IgG2, IgG3 and IgG4 proteins (Sigma-Aldrich, Missouri, USA) were used to standardise assays. Proteins were supplied lyophilised and reconstituted in distilled H_2O according to the manufacturer's instructions.

7.2.4 Enzyme-linked immunosorbent assay

Fifty microlitres of Ag85B (10 μg/ml) and IgG subclass protein at various concentrations (20 μg/ml, 10 μg/ml, 5 μg/ml, 2 μg/ml, 1 μg/ml, 0.2 μg/ml, 0.02 μg/ml) in 0.05 M $NaHCO_3$ was allowed to bind to duplicate wells of ELISA plates (Immulon 4HSX, Thermosystems, USA) at 37°C for 2 hours, and plates were stored at 4°C overnight. After three washes with PBS/0.05% (v/v) Tween-20, PBS (phosphate buffered saline; 0.01 M phosphate buffer, 0.0027 M potassium chloride, 0.137 M sodium chloride, pH7.4) containing 5% (w/v) BSA was added to the plates and incubated for 2 hours at 37°C to block free sites. Following this, plates were washed thrice with PBS/0.05% (v/v) Tween-20 to remove unbound protein. Sera diluted in 1% (w/v) BSA/0.05%

(v/v) Tween-20 in PBS was added to the plates and incubated for 2 hours at 37°C, and then stored at 4°C overnight. Plates were washed thrice with PBS/0.05% (v/v) Tween-20, as before. Mouse monoclonal antibodies (mAbs) specific for each of the human IgG subclasses (HP6001, anti-IgG1; HP6002, anti-IgG2; HP6047, anti-IgG3; HP6023, anti-IgG4; Sigma-Aldrich, Missouri, USA) were added at saturating concentrations (1:5 000 dilution), incubated for 2 hours at 37°C, and then stored at 4°C overnight. Plates were washed thrice with PBS/0.05% (v/v) Tween-20, as before. Horseradish peroxidase (HRP)-labelled goat anti-mouse IgG (CALTAG laboratories, Burlingame, USA) was added at pre-determined optimal concentrations of 1:1 000 for anti-IgG1 and anti-IgG4 and 1:5 000 for anti-IgG2 and anti-IgG3, and plates incubated for 2 hours at 37°C. Plates were washed thrice with PBS/0.05% (v/v) Tween-20 and developed with 100 µl of tetramethylbenzidine substrate (TMB, Kirkegaard & Perry Laboratories Inc., Gaithersburg, USA). After 10 minutes at room temperature, colour development was stopped by adding 100 µl of 1 M H_2SO_4. Plates were read at 450 nm using a bench top microplate reader (Bio-Rad laboratories, Hercules, USA). Sera were analysed at four different dilutions and activity expressed as optical density relative to the standard included in each assay.

7.2.5 Statistical analysis

Statistical analysis was performed using the GraphPad Prism version 4.00 for Windows software package (GraphPad Software, San Diego, California, USA, http://www.graphpad.com). Paired t-tests were used to determine the significance between patient status and antibody responses.

7.3 RESULTS

7.3.1 Optimisation of antibody concentrations

The optimal concentration of anti-IgG subclass mAb and goat HRP-labelled anti-mouse IgG was determined by calculating the signal-to-noise ratio of six dilutions of each of the four anti-IgG subclass mAbs and eight dilutions of secondary antibody against 0.25 μg/well of IgG subclass protein specific for each of the mAbs. Results showed that the optimal dilution for anti-IgG1, anti-IgG2, anti-IgG3 and anti-IgG4 was 1:5000, whereas the optimal dilution for the secondary antibody was 1:5 000 for anti-IgG2 and anti-IgG3, and 1:1 000 for anti-IgG1 and anti-IgG4 (data not shown).

7.3.2 Standard curve

Various concentrations (0.5 μg-0.0001 μg) of purified IgG subclass protein were probed with the previously determined optimal concentration of subclass-specific mAb and the optimal concentration of secondary antibody. Plotting OD_{450} against protein concentration on a semi-log scale allowed the generation of straight lines with correlation co-efficients ranging between 0.94 and 0.98 (Figure 7.1). The gradient of each of these curves was used to standardise assays.

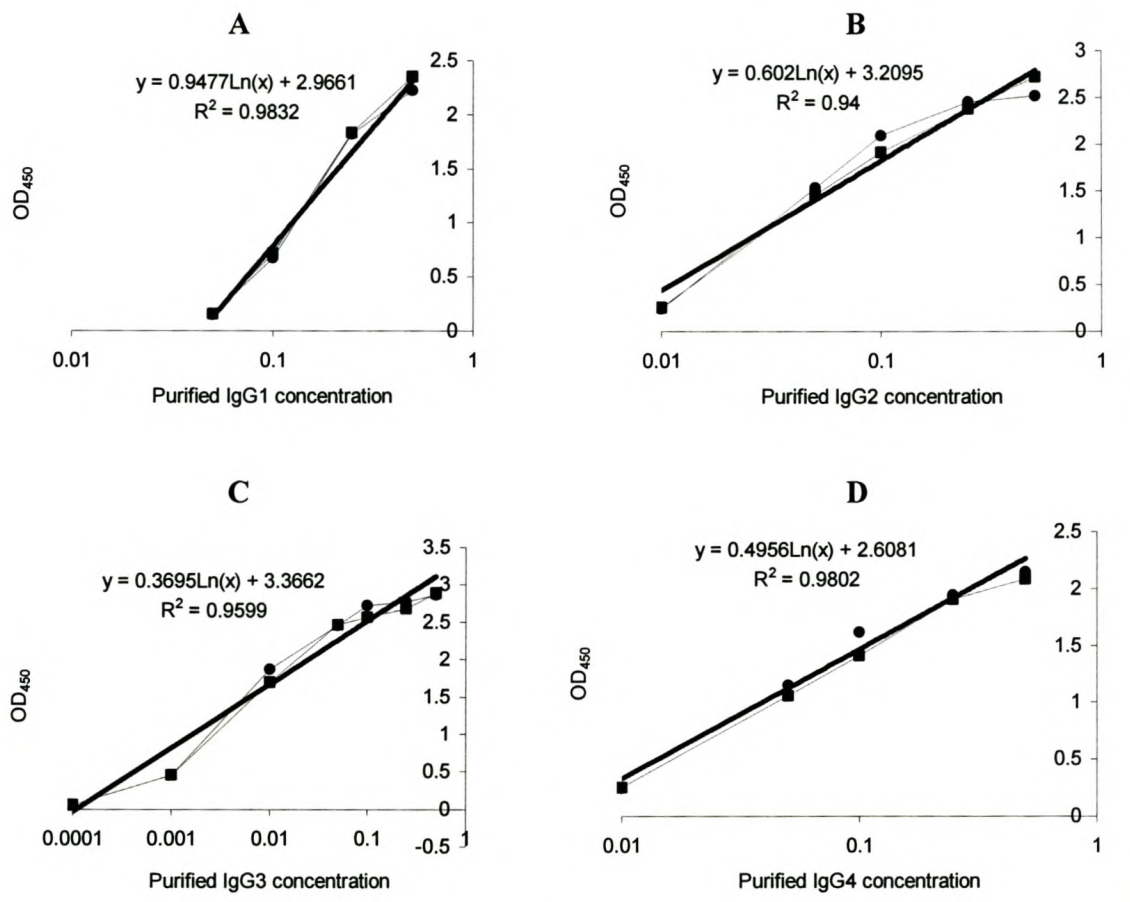

Figure 7.1 Reactivity of IgG subclass proteins with their specific antibodies. Various concentrations of IgG1 purified protein (A), IgG2 purified protein (B), IgG3 purified protein (C) and IgG4 purified protein (D) were probed with optimal concentrations of the corresponding mouse monoclonal antibody. Standard curves were obtained by plotting protein concentration against OD_{450} on a semi-log scale. The circles and squares represent duplicate experiments. The thicker line represents the linear trendline generated.

7.3.3 IgG subtyping

Preliminary experiments were done with sera collected from ten TB patients at diagnosis and after four weeks of compliant therapy. Pilot experiments with these patients served to validate the assay for future experiments with "responders" and "nonresponders". Ag85B-IgG subclass levels showed inter-patient variability, with the difference in antibody levels between diagnosis and after four weeks of therapy being statistically non-significant (IgG1 (p=0.7337), IgG2 (p=0.8340), IgG3 (p=0.9657) and IgG4 (p=0.2888)) (Figure 7.2). Despite this, it was decided to measure IgG subtype levels in "responders" and "nonresponders" identified by smear conversion after two months of antimycobacterial chemotherapy. These patients were matched for age, sex and extent of disease. These two groups were analysed to determine whether their IgG subclass levels at diagnosis and/or after fours weeks of antimycobacterial chemotherapy could predict smear status after two months of treatment. Slight differences between "responders" and "nonresponders" were noted, however, these were not statistically significant. At diagnosis, IgG1 (p=0.3538) and IgG4 (p=0.4414) were higher for "responders" compared to "nonresponders". IgG3 (p=0.5446) was slightly higher in "nonresponders" compared to "responders", while the levels of IgG2 (p=0.9479) was almost the same in the two groups (Figures 7.3). After four weeks of treatment, levels of IgG1 were still higher in "responders" (p=0.5786) and levels of IgG3 were still higher in "nonresponders" (p=0.7778). "Responders" appeared to have slightly higher levels of IgG2 (p=0.8760), while "nonresponders" had slightly higher levels of IgG4 (p=0.6025) after four weeks of treatment. Dissecting the antibody response at diagnosis and after four weeks of treatment for the "responder" and "nonresponder" group separately did not show a pattern in IgG isotype production (Figure 7.5 and Figure 7.6). For some patients a decrease in Ag85B-specific IgG subtype levels was observed, while for others patients, levels of the same IgG subtypes increased during treatment. Dissecting the IgG subtype response

for individual patients did not show any significant patterns in IgG subtype responses for individual patients. Analysis of the difference between IgG subclass levels at diagnosis and after four weeks antimycobacterial chemotherapy for each of the patients individually also showed no significant profile difference for "responders" and "nonresponders" (Figure 7.7).

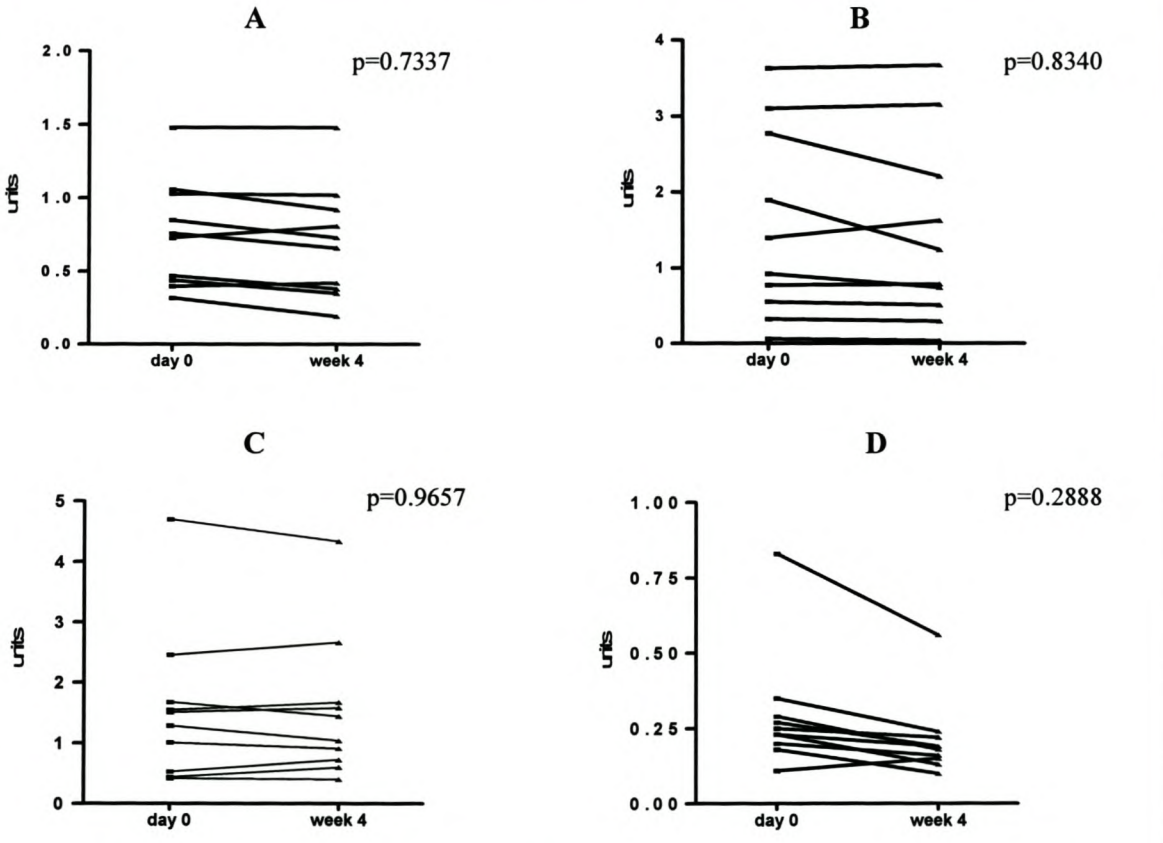

Figure 7.2 Pilot IgG subtyping study. Ag85B-specific IgG1 (A), IgG2 (B), IgG3 (C) and IgG4 (D) of ten TB patients were measured at diagnosis and after four weeks of antimycobacterial chemotherapy. The y-values represent units relative to the standard included in each assay. Each point represents the mean of duplicate samples.

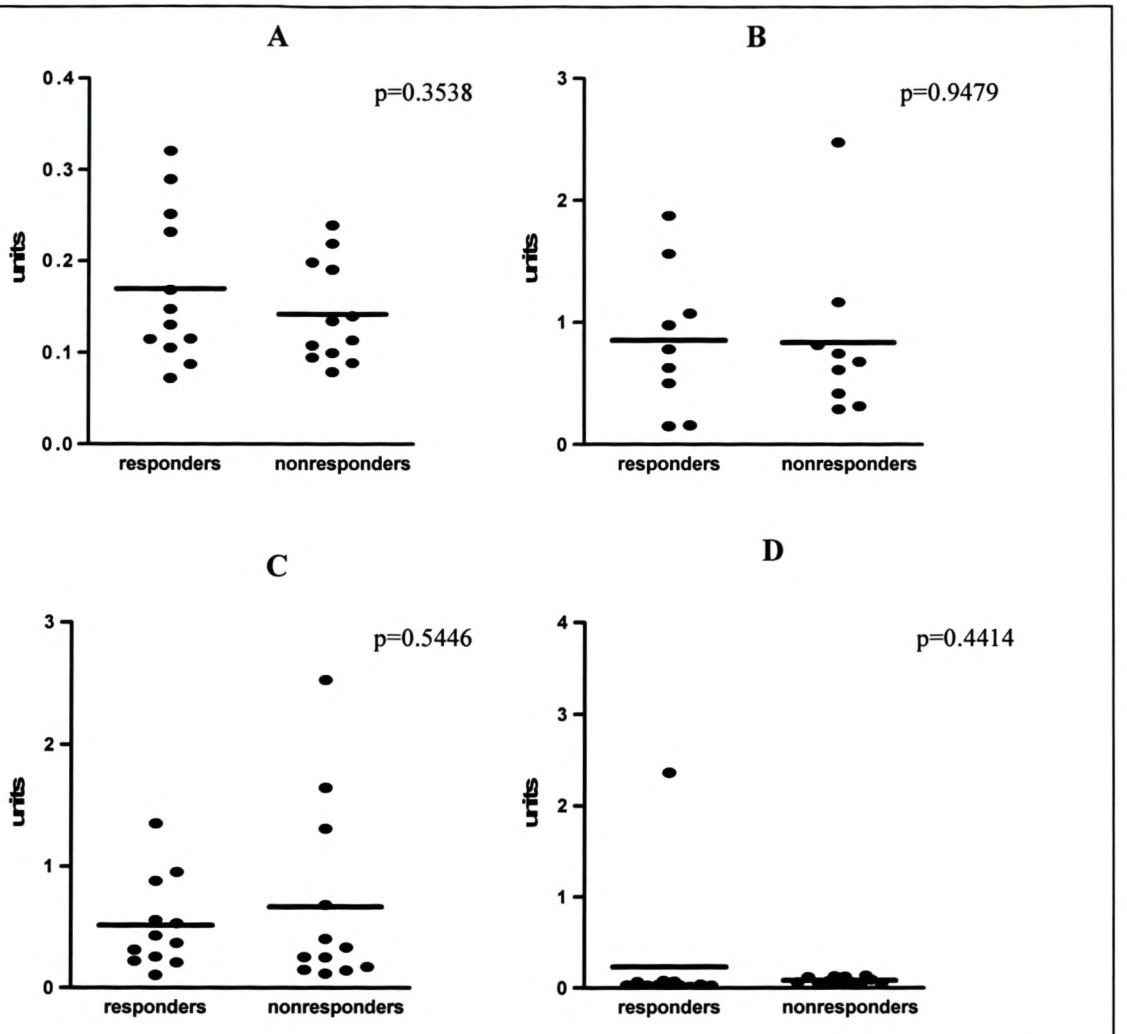

Figure 7.3 IgG subclass levels of "responders" and "nonresponders" at diagnosis. Ag85B-specific IgG1 (A), IgG2 (B), IgG3 (C) and IgG4 (D) of patients who were smear negative (responders) or smear positive (nonresponders) after two months of antimycobacterial chemotherapy. The y-values represent units relative to the standard included in each assay. Each point represents the mean of duplicate samples and two independent assays.

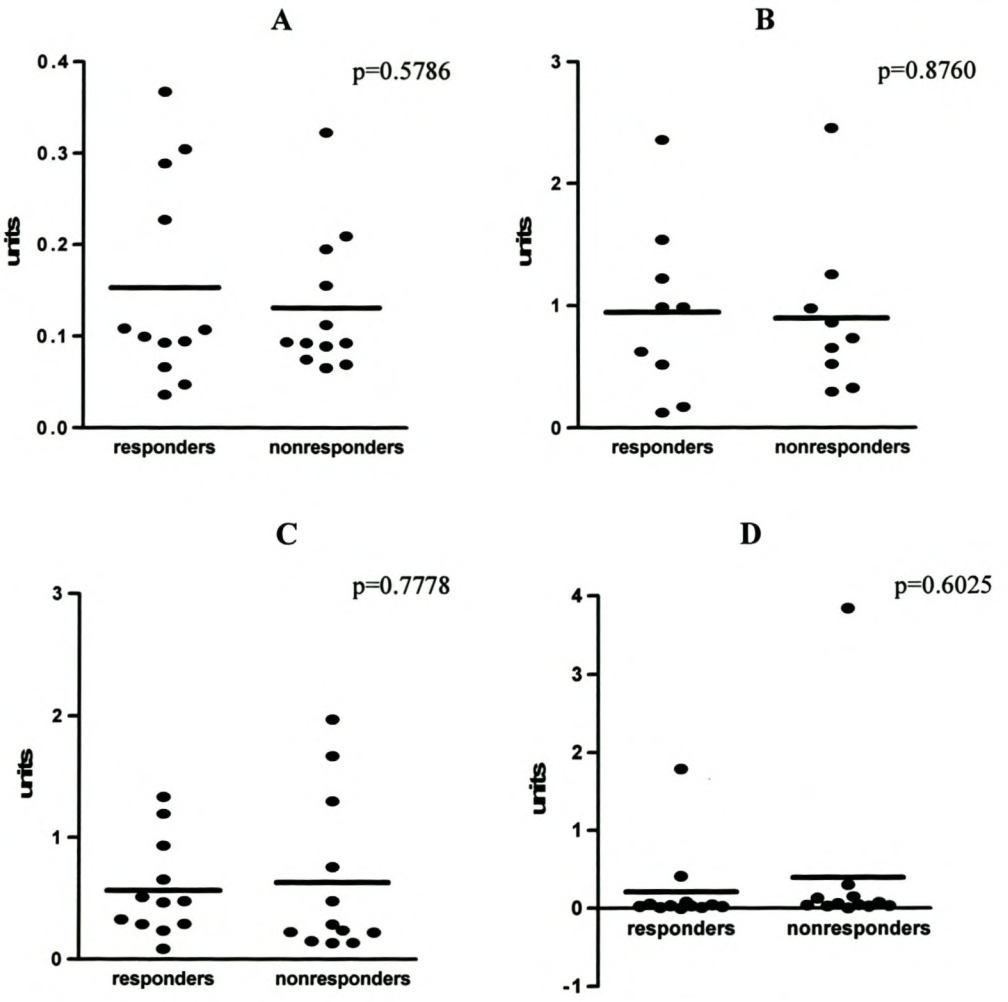

Figure 7.4 IgG subclass levels of "responders" and "nonresponders" after four weeks of antimycobacterial chemotherapy. Ag85B-specific IgG1 (A), IgG2 (B), IgG3 (C) and IgG4 (D) of patients who were smear negative (responders) or smear positive (nonresponders) after two months of antimycobacterial chemotherapy. The y-values represent units relative to the standard included in each assay. Each point represents the mean of duplicate samples and two independent assays.

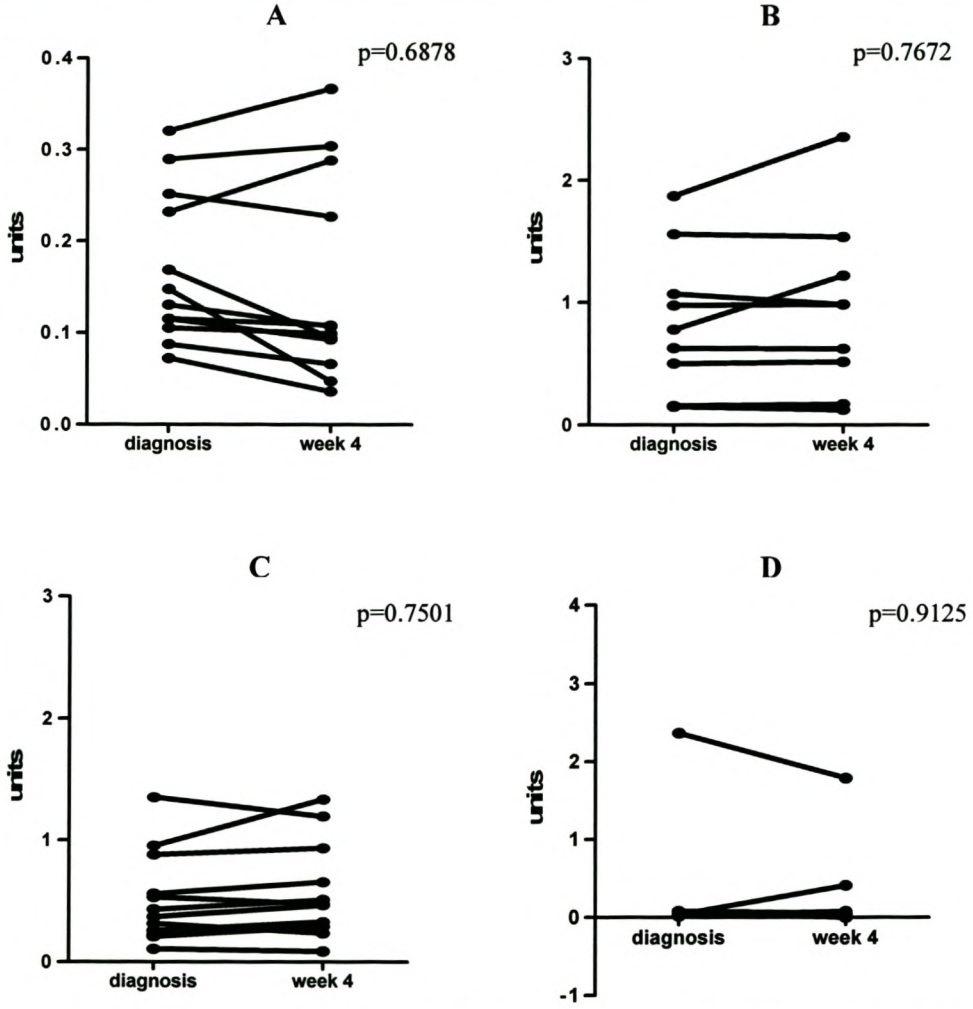

Figure 7.5 IgG subclass levels of "responders" at diagnosis and after four weeks of antimycobacterial chemotherapy. Ag85B-specific IgG1 (A), IgG2 (B), IgG3 (C) and IgG4 (D) of patients who were smear negative (responders) after two months of antimycobacterial chemotherapy. The y-values represent units relative to the standard included in each assay. Each point represents the mean of duplicate samples and two independent assays.

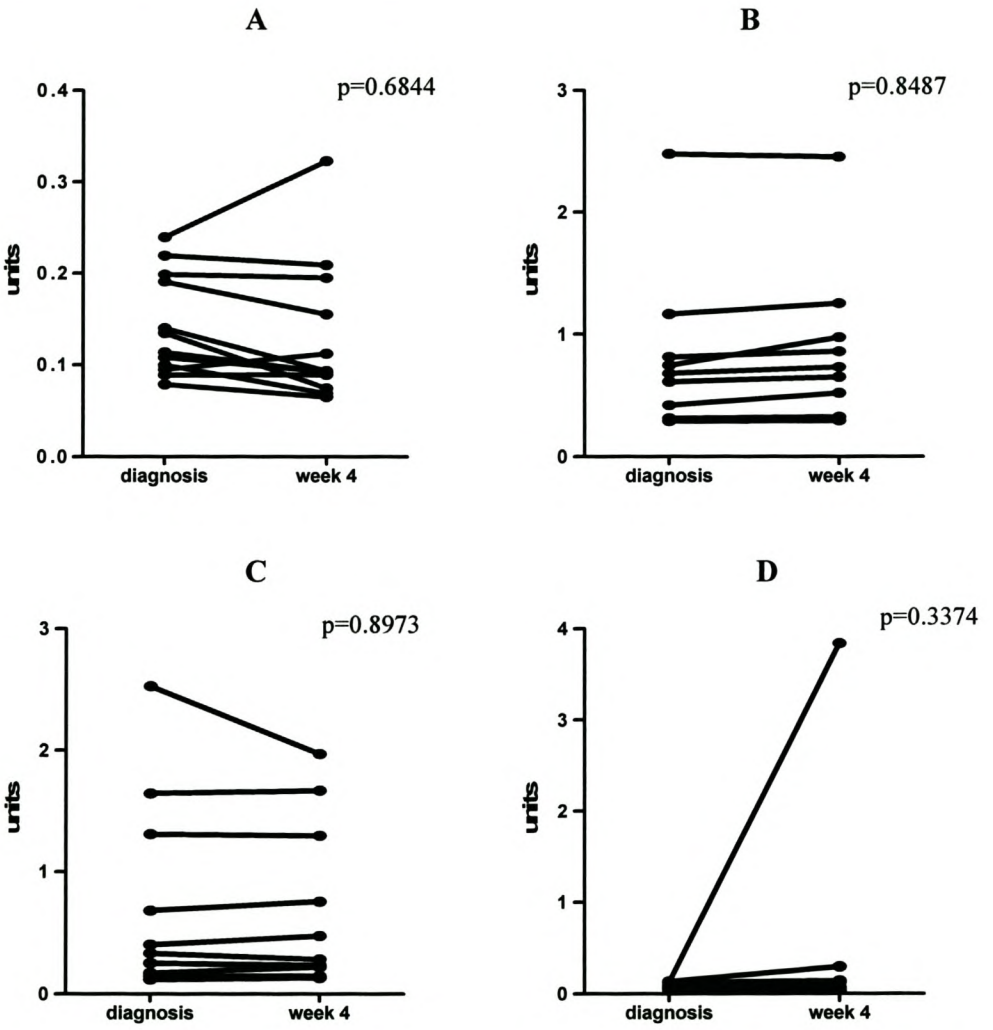

Figure 7.6 IgG subclass levels of "nonresponders" at diagnosis and after four weeks of antimycobacterial chemotherapy. Ag85B-specific IgG1 (A), IgG2 (B), IgG3 (C) and IgG4 (D) of patients who were smear positive (nonresponders) after two months of antimycobacterial chemotherapy. The y-values represent units relative to the standard included in each assay. Each point represents the mean of duplicate samples and two independent assays.

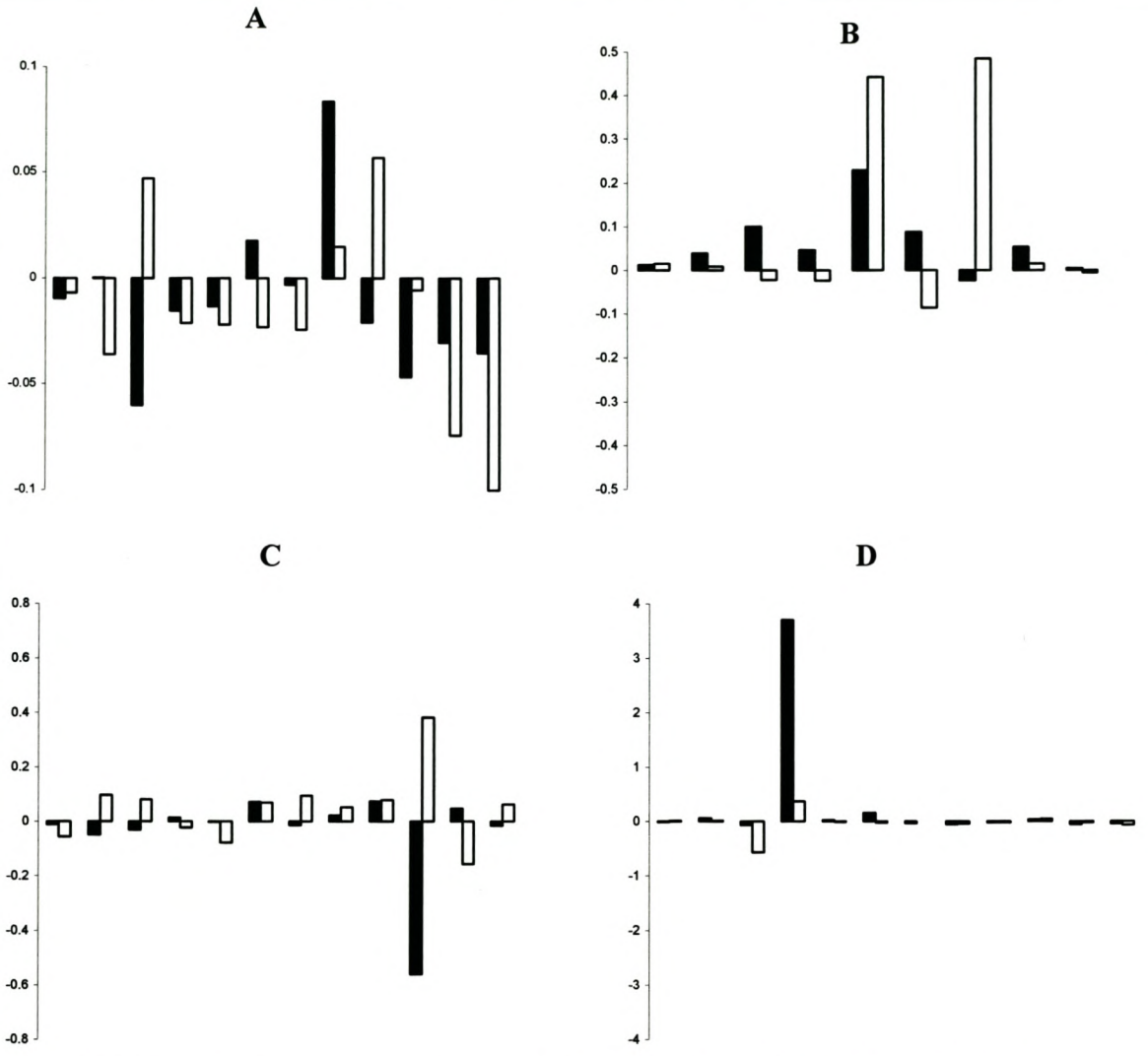

Figure 7.7 Difference in IgG subclass levels at diagnosis and after four weeks of antimycobacterial chemotherapy. The increase in Ag85B-specific IgG1 (A), IgG2 (B), IgG3 (C) and IgG4 (D) levels between diagnosis and after four weeks of antimycobacterial chemotherapy is plotted for each matched nonresponder (shaded bars) and responder (clear bars) pair. Each bar represents the mean of duplicate samples and two independent assays.

7.4 DISCUSSION

Cell-mediated immunity (CMI) confers protection against TB (Flynn and Chan, 2001), while the role of humoral immunity is controversial (Glatman-Freedman and Casadevall, 1998). Recent reports suggest that antibodies may play a protective role in TB (Glatman-Freedman, 2000; Pethe *et al.* 2001; Teitelbaum *et al.* 1998). A direct link between CMI and humoral immunity was suggested when Hussain and co-workers demonstrated that opsonizing antibodies (IgG1) may aggravate disease pathology by upregulating proinflammatory cytokines (Hussain *et al.* 2000, 2001). Regardless of their role in disease pathogenesis, antibodies are markers of antigen expression *in vivo*, and therefore may reflect the metabolic state of the bacillus in the host. Patients vary in their response to treatment, which may be due to different metabolic activities of tubercle bacilli during antimycobacterial chemotherapy. Antibodies may reflect the differences in antigenic make-up of bacilli during these different metabolic states, allowing differentiation between patients experiencing successful treatment and those who will relapse. Furthermore, the development of antibodies to cytoplasmic antigens of *M. tuberculosis* may confirm successful killing of bacilli by antimycobacterial drugs. We decided to assess Ag85B-specific IgG1, IgG2, IgG3 and IgG4 levels of two groups of TB patients who smear converted (responders), or who remained smear positive (nonresponders) after two months of antimycobacterial chemotherapy. These patients were fully compliant during treatment, HIV negative, had drug sensitive bacilli, and did not have any other disease known to affect the immune system.

Analysis of IgG subclass levels at diagnosis, after four weeks of antimycobacterial chemotherapy, and the difference in antibody levels between week four and diagnosis showed no significant difference between "responders" and "nonresponders". Sousa *et al.* (2000) also failed

to see a difference in IgG levels to mycobacterial antigens during treatment. Patient-to-patient variation underlies humoral immunity (Lyashchenko *et al.* 1998a) and probably explains the lack of significant differences between the two groups. Several factors could explain the humoral heterogeneity, including genetic variability between patients, *M. tuberculosis* strain differences, extent of disease at diagnosis, bacterial load and antimycobacterial chemotherapy (Lyashchenko *et al.* 1998a). Patients investigated in this study were smear positive, had primary TB and were all at the same stage of therapy, disputing the suggestion that humoral heterogeneity is due to bacterial load and antimycobacterial chemotherapy. Previous findings by us and others support the involvement *M. tuberculosis* strain type (Pheiffer *et al.* 2002), host genetics (Bothamley *et al.* 1989) and extent of disease (Pheiffer *et al.* 2002; Samanich *et al.* 1998) in the host humoral immune response.

Sánchez-Rodriguez *et al.* (2002) reported that positive anti-Ag85 antibody titres correlated with a good outcome. Our conflicting results are possibly due to the fact that we measured IgG subtype levels to recombinant Ag85B whereas Sanchez-Rodriguez *et al.* quantified native Ag85 complex-specific IgG. Ag85 consists of Ag85A, Ag85B and Ag85C, and perhaps all of these should be investigated. Furthermore, recombinant proteins expressed in *E. coli* may not contain the same epitopes as native Ag85B or recombinant Ag85B expressed in mycobacterial hosts (Triccas *et al.* 1998). Studies have shown that many mycobacterial antibodies are complexed to antigens in immune complexes (Bhattacharya *et al.* 1986) and maybe these should be investigated. Raja *et al.* (1995) reported that the kinetics of circulating immune complexes (CICs) changes during treatment and these could possibly serve as markers for treatment efficacy. However, more recently Sousa *et al.* (2000) showed no correlation between circulating IgG antibodies to several *M. tuberculosis* antigens (38 kDa, short term culture filtrate, DAT or

LAM) or CICs with treatment. We analysed patients categorised into "responders" and "nonresponders" using smear culture conversion after two months of antimycobacterial chemotherapy. This classification of patients may be flawed, and using a more accurate distinction, such as comparing antibody levels of patients who relapse to antibody levels of patients who are successfully cured and do not relapse may yield interesting differences between the two groups. However, this study was designed to investigate whether patient antibody levels early during treatment could predict treatment response.

Studies have shown a reduction in antigenic load in sputum (Wallis *et al.* 1998) and in blood (Sethna *et al.* 1998) in response to treatment, suggesting that measurement of mycobacterial antigen may be more useful than measurement of antibody levels. Furthermore, calculating the ratio of secretory antigens (indicative of active growth) and cytoplasmic antigens (indicative of bacterial lysis) in patient sera may be a better indicator of treatment response and therefore outcome than measurement of antibody to a single antigen (Sethna *et al.* 1998). Future work should investigate the difference in secreted and cytoplasmic antigens from a number of samples for each patient and monitor the ratio of IgG and CICs to these antigens.

This study has shown that Ag85B-specific IgG subclass antibodies do not correlate with smear status, and are therefore not useful in predicting treatment outcome. However, mycobacterial antigen and antibody detection offers several advantages over microscopic detection, bacterial culture, and culturing of host cells, and should therefore be explored further.

7.5 APPENDIX

Preliminary experiments were performed to optimize conditions for profiling the humoral immune response of patients undergoing chemotherapy.

7.5.1 Optimization of antigen and secondary antibody concentration

The optimal concentration of recombinant mycobacterial antigens (Ag85B, MPT64, Hsp65 and α-crystallin, Lionex Diagnostics and Therapeutics, Braunschweig, Germany) and secondary antibody (HRP-labelled goat anti-human IgE, IgG, IgG1, IgG4, IgM, CALTAG laboratories, Burlingame, USA) was determined in an indirect ELISA. Briefly, antigen diluted in 0.05 M $NaHCO_3$ (concentrations ranging from 0.05 μg/well to 0.5 μg/well) was allowed to bind to duplicate wells of ELISA plates (Nunc, Roskilde, Denmark) overnight. The following day, wells were washed six times with PBS containing 0.05% (v/v) Tween-20. Unbound sites were blocked with 2% (w/v) non-fat milk powder (Clover, Roodepoort, South Africa) in PBS/0.05% (v/v) Tween-20 (blocking buffer) for 2 hours at 37°C. Thereafter wells were washed as before and serum from a Mantoux positive laboratory volunteer was added for 2 hours at 37°C. After washing wells, secondary antibodies were diluted according to the manufacturers suggestions and used at dilutions ranging from 1:1 000 to 1:15 000. Plates were incubated for 1 hour at 37°C, washed with PBS/0.05% (v/v) Tween-20 and 100 μl of tetramethylbenzidine substrate (TMB, Kirkegaard & Perry Laboratories Inc., Gaithersburg, USA) was added. After 10 minutes at room temperature, colour development was stopped by adding 100 μl 1 M H_2SO_4. Plates were read at 450 nm using a bench top microplate reader (Bio-Rad laboratories, Hercules, USA). In two independent experiments, we found that the optimal dilution of secondary antibodies was 1:1 000 for all four antigens, although the optimal concentration of antigen varied (Table 7.1).

Table 7.1. Optimal antigen concentrations.

	Concentration (μg/well)
Ag85B	0.25
MPT64	0.005
Hsp65	0.125
α-crystallin	0.5

7.5.2 Inter-assay variation

To determine variability between experiments, a series of serum dilutions (1:50, 1:100, 1:200, 1:500, 1:1 000, 1:1 500) of the Mantoux positive laboratory donor used in section 7.5.1 was probed against each of the four antigens at pre-determined optimal concentrations. Anti-human IgG was used as the secondary antibody. Slight differences in absolute OD_{450} readings between experiments were observed, although the overall trend remained the same. Results of experiments performed on different days were consistent (Figure 7.8). In an independent experiment we found that the intra-assay and inter-assay co-efficient of variation was less than 5% and 10%, respectively (data not shown).

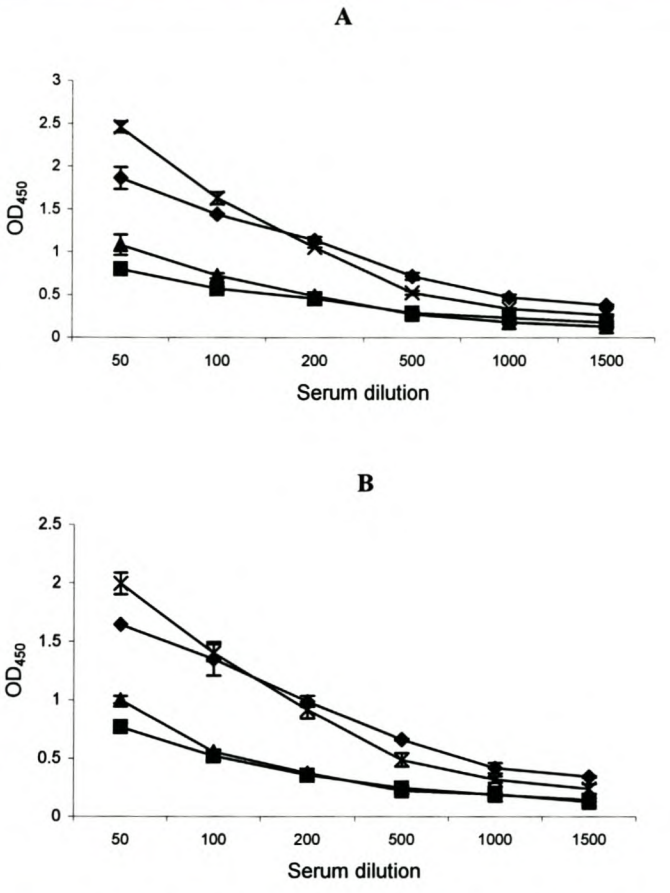

Figure 7.8 Inter-assay analysis of the internal standard. IgG levels of the Mantoux positive laboratory donor to Ag85B (◆), MPT64 (■), Hsp65 (×) and α-crystallin (▲). Experiments A and B were done on two different days. Results are expressed as the mean between duplicate samples ± standard deviation from the mean.

7.5.3 Validation with patient sera

The IgG and IgM responses of ten TB patients were measured. Antibody levels were measured in sera collected from patients at diagnosis and after four weeks of antimycobacterial chemotherapy. All patients were 90% compliant during this time (Table 7.2). Probing three different sera dilutions of each of the ten patients against Ag85B, MPT64, Hsp65 and α-crystallin showed no significant difference in IgG levels at diagnosis and after four weeks of treatment (Figure 7.9).

Table 7.2. Compliance, as measured by drug taking, of TB patients analysed.

Patient No.	Diagnosis	Week 4	Doses	Missed Doses
4831	12/07/2000	08/08/2000	20/20	
2928	07/07/1999	03/08/1999	20/20	
4959	08/11/2000	05/12/2000	20/20	
4536	07/01/2000	03/02/2000	20/20	
4715	24/05/2000	20/06/2000	19/20	16/06/2000
2900	30/06/2000	27/07/2000	20/20	
2901	30/06/1999	27/07/1999	19/20	14/07/1999
5033	29/01/2001	23/02/2001	20/20	
4614	09/03/2000	05/04/2000	20/20	
4816	05/07/2000	01/08/2000	20/20	

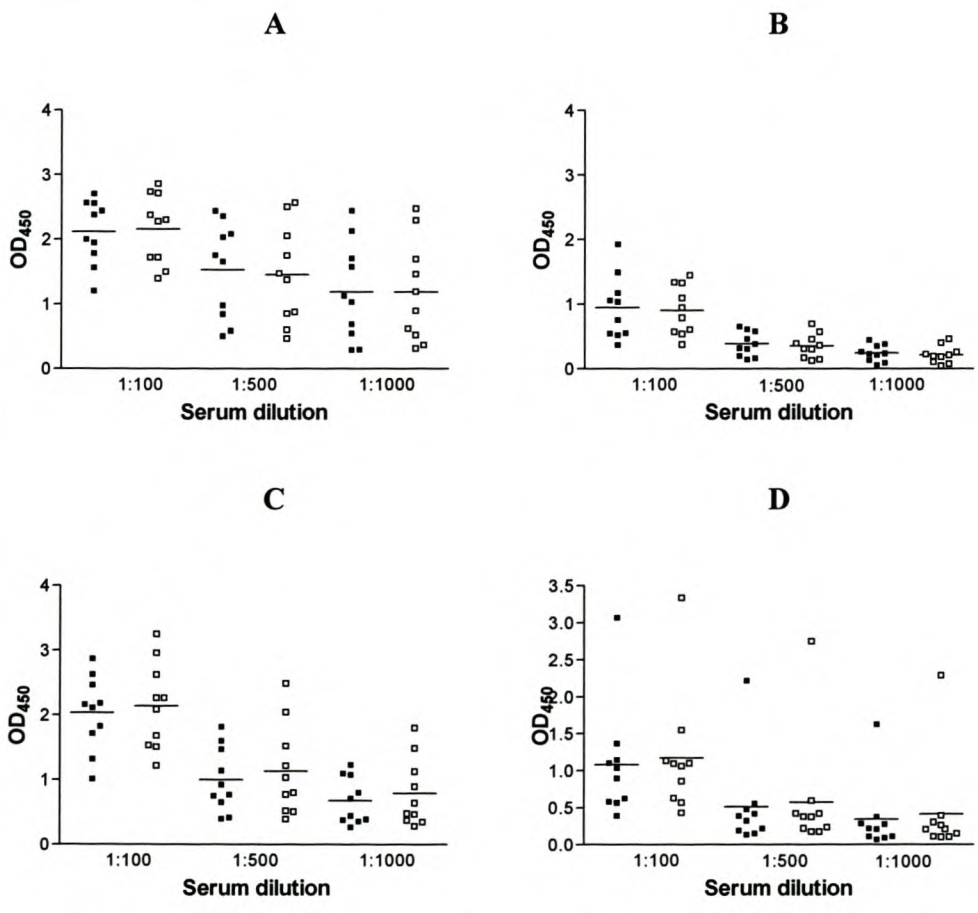

Figure 7.9 Serum IgG levels of ten TB patients. Sera at diagnosis (solid blocks) and after four weeks of treatment (open blocks) were diluted 1:100, 1:500 and 1:1 000 and probed against recombinant Ag85B (A), MPT64 (B), Hsp65 (C) and α-crystallin (D). Each point represents the mean of duplicate samples.

To simplify the analysis of patient IgG levels, OD_{450} readings were plotted against sera dilutions on a semi-log scale, a linear relationship between OD_{450} readings and sera dilutions was assumed and the activity of each patient serum sample expressed as one unit, relative to the internal standard (Mantoux positive laboratory donor). It was decided to use seven different patient sera dilutions, since using only three dilutions did not show any significant differences (Figure 7.9). As before, IgG levels of patients to Ag85B, MPT64, Hsp65 and α-crystallin at diagnosis and after four weeks of treatment was not significantly different (Ag85B, p=0.0978; MPT64, p=0.5698; Hsp65, p=0.7912, α-crystallin, p=0.7078) (Figure 7.10). IgM levels against Ag85B, MPT64, Hsp65 and α-crystallin were measured using the same range of serum dilutions as used for IgG measurement. IgM levels were higher after four weeks of treatment, although the difference between the response at diagnosis and after four weeks of treatment was not statistically significant (Ag85B, p=0.6394; MPT64, p=0.7356; Hsp65, p=0.7694; α-crystallin, p=0.1091) (Figure 7.11).

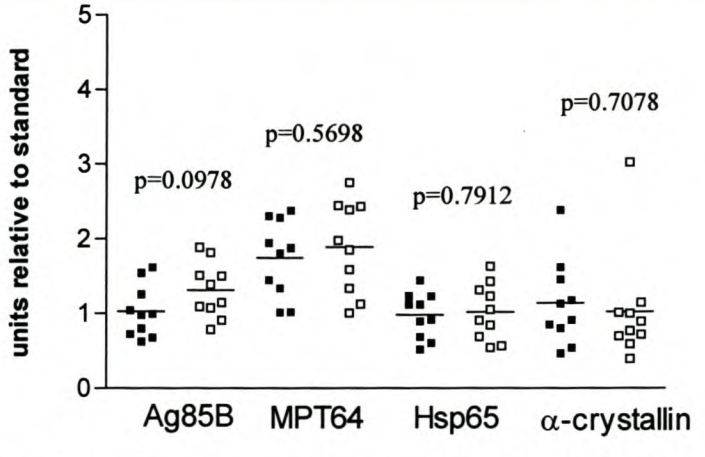

Figure 7.10 IgG responses to mycobacterial antigens. Levels of serum IgG to recombinant antigens of *M. tuberculosis* at diagnosis (solid blocks) and after four weeks of treatment (open blocks) are expressed as units of activity relative to a reference calibrator. Each point represents the mean of duplicate samples.

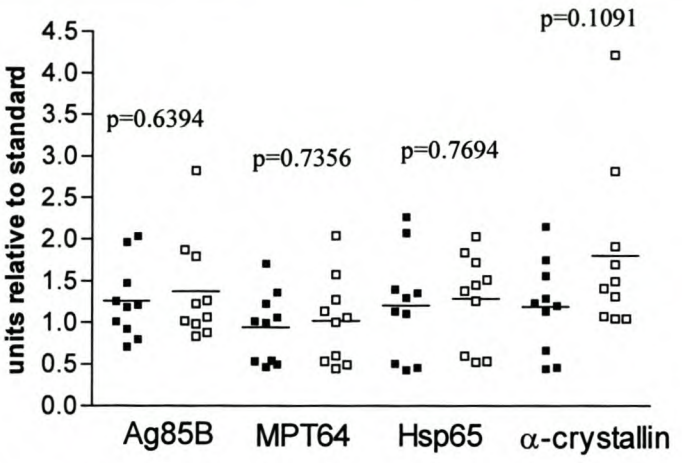

Figure 7.11 IgM responses to mycobacterial antigens. Levels of serum IgM to recombinant antigens of *M. tuberculosis* at diagnosis (solid blocks) and after four weeks of treatment (open blocks) are expressed as units of activity relative to a reference calibrator. Each point represents the mean of duplicate samples.

7.5.4 Total IgG versus IgG subclasses

It has been suggested that measurement of IgG subclasses may be more informative than measurement of total IgG (Gibson *et al.* 1987; Hussain *et al.* 1995). This hypothesis was tested by measuring antibody responses to *M. tuberculosis* whole-cell lysate proteins using HRP-labelled anti-human IgG (CALTAG laboratories, Burlingame, USA), HRP-labeled anti-human IgG1 (CALTAG laboratories) and HRP-labelled anti-human IgG4 (CALTAG laboratories) as secondary antibodies. No significant difference in total IgG was observed, although IgG1 levels varied between patients, supporting the hypothesis that IgG subtyping is more useful than measuring total IgG (Figure 7.12). No whole-cell lysate specific IgG4 was detected in any of the patients. No Ag85B-specific IgG1 nor IgG4 were detected in any of these patients (data not shown). These results suggest that future subtyping should be done using mouse mAbs specific for human IgG1, IgG2, IgG3 and IgG4 in a sandwich ELISA which is more sensitive than the

indirect ELISA. ELISA plates with a higher binding capacity should also be used (Hussain *et al.* 1986a, 1986b).

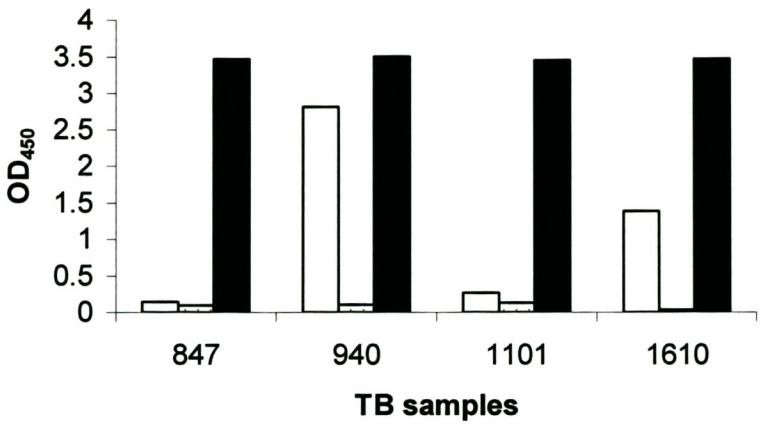

Figure 7.12 IgG responses to *M. tuberculosis* whole-cell lysate proteins. Levels of IgG1 (clear), IgG4 (dotted), and total IgG (shaded) were measured in sera from TB patients.

Chapter 8

GENERAL DISCUSSION AND CONCLUSION

8.1 Introduction

Despite extensive research, TB remains a devastating disease associated with high morbidity and mortality. The development of new diagnostics, vaccines and antimycobacterial drugs, is dependent on understanding the mechanisms of pathogenesis of *M. tuberculosis*. TB is a complex disease and its expression depends on both host and pathogen related factors. A variety of innate and acquired host factors have been described that affect the variability in the response to *M. tuberculosis* infection and the severity of disease (Bellamy and Hill, 1998b). However, very little is known about the role of the pathogen in disease establishment and progression.

8.2 Protein expression and antigen presentation patterns of clinical isolates

In this thesis one aspect of the biological significance of genotypic variation between *M. tuberculosis* strains was investigated by comparing protein expression profiles and antigen recognition patterns of clinical strains. The results described in this thesis reveal some insights into immune evasion mechanisms, strain prevalence, failure of serodiagnostic tests and exogenous reinfection.

8.2.1 Strain prevalence

Molecular epidemiological studies have identified *M. tuberculosis* strains families that are more "successful" or "fit" than others, as assessed by their ability to spread in a community (Warren *et al.* 2000). The most well-known example of this is the Beijing family, a family of *M. tuberculosis* strains thought to have originated in Asia (van Soolingen *et al.* 1995). *M. tuberculosis* strains belonging to the Beijing family are distributed worldwide and are able to spread in large clonal clusters (Glynn *et al.* 2002). It is speculated that these strains have an

advantage over other less prevalent isolates, and that identification of the factors responsible would aid understanding of the success of the Beijing strain family (Bifani *et al.* 2002).

To investigate this hypothesis we compared protein expression patterns and antigen recognition profiles of a strain belonging to the Beijing family to a less prevalent clinical isolate. The differential protein expression and antigen recognition profiles observed for these strains in our patients may relate to strain prevalence, possibly due to immune evasion. The ability to cause disease due to immune evasion mediated by differential protein expression patterns has been reported for a number of other pathogens (Smith *et al.* 2002), and our results suggest that a similar mechanism for disease establishment may exist for *M. tuberculosis*, and may relate to strain prevalence.

Development of techniques such as genome-directed priming (Talaat *et al.* 2000), protein microarrays (Cahill, 2001) and proteomics using isotope-coded affinity tags (ICAT) (Gygi *et al.* 2000) should facilitate the analysis of global gene expression *in vivo*, an important requirement to validate the significance of our *in vitro* findings. Moreover, analysis of additional *M. tuberculosis* genotypes may reveal expression profiles characteristic of specific strain families, and possibly identify strain-specific factors that may be tested in animal models to determine their role in disease pathogenesis.

8.2.2 Implications for serodiagnosis of TB

The development of antibody detection-based tests for the diagnosis of TB has received extensive attention, however, no test has become available for general clinical use (Bothamley, 1995; Chan *et al.* 2000). This study has provided some clues about the failure to develop

serodiagnostic tests with sufficient sensitivity, by showing that protein expression by *M. tuberculosis* strains is dynamic, varying with growth stage and to a certain extent with strain type. Extensive humoral heterogeneity between TB patients, possibly due to differential protein expression by *M. tuberculosis* strains or due to host factors may further complicate the development of serodiagnostic tests.

The most significant finding of this study was decreased expression of PstS1 by the Beijing strain compared to the other clinical strain and H37Rv. PstS1 is a mycobacterial phosphate binding protein that has been extensively evaluated as a possible serodiagnostic candidate (Bothamley, 1995; Chan *et al.* 2000). Failure of certain *M. tuberculosis* strains to express PstS1, or decreased expression, may have important implications for serodiagnosis of TB and may explain the absence of PstS1-specific antibody levels in some TB patients.

8.2.2.1 Mechanism of differential PstS1 expression

It has been reported that IS*6110* insertions occur predominantly in coding regions of *M. tuberculosis* (Sampson *et al.* 1999; Warren *et al.* 2000), thereby creating "natural gene knockouts". Reviewing the literature available on IS*6110* insertion (Beggs *et al.* 2000; Sampson *et al.* 1999; Warren *et al.* 2000), showed that reduced expression of PstS1 by the Beijing strain was not due to IS*6110* insertion. Decreased expression of PstS1 was also shown not to be due to *pstS1* sequence variations, suggesting that expression of PstS1 is probably regulated at the transcriptional and/or translational level. Transcriptional and/or translational control is an adaptive strategy available to a number of other pathogens (Weiser *et al.* 1989; Zhang and Wise, 2001). Negligible genetic diversity within coding regions of *M. tuberculosis* (Musser *et al.* 2000;

Sreevatsan *et al.* 1997) highlights the importance of transcriptional and/or translational regulation in protein expression by *M. tuberculosis*.

8.2.3 Vaccine and drug development

Investigation of protein expression and antigen recognition profiles of *M. tuberculosis* strains during conditions which are suggested to be host-relevant revealed possible vaccine and drug candidates to target the persistent phase of *M. tuberculosis* infection. For example, Rv2557 and Rv2558, which were upregulated during nutrient deprived and stationary growth in all clinical strains, is expressed in human tuberculous granulomas (Fenhalls *et al.* 2002), and are potential vaccine candidates. The finding of differential antigen recognition profiles may have important implications for the development of subunit and DNA vaccines.

8.3 Antibody responses of TB patients

8.3.1 Potential for serodiagnosis of TB

To investigate the potential of serodiagnosis in our community, we measured IgG levels to three mycobacterial antigens that have been extensively used as possible serodiagnostic antigens (Bothamley, 1995; Chan *et al.* 2000). The sensitivity of the assay was significantly improved by combining all three antigens, rather than measuring reactivity to individual antigens, thereby confirming patient-to-patient variation in antigen recognition, and supporting the development of tests based on multi-antigen cocktails. This study has also shown that serodiagnostic tests may be prone to false-positivity due to persistence of antibodies after the previous TB episodes.

8.3.2 Potential as surrogate marker to predict treatment outcome

Drug development is hindered by the lengthy duration of clinical trials and associated high costs. Antimycobacterial chemotherapy affects the antibody profile of TB patients (Bothamley *et al.* 1992a; Imaz and Zerbini, 2000), supporting the potential of antibody levels as surrogate markers for treatment outcome. In this study we explored the possibility of using patient antibody levels after four weeks of treatment as a surrogate marker to predict treatment response, measured by smear status after two months of treatment. Although Ag85B-specific IgG levels did not correlate with treatment response, our work has demonstrated the advantages of using antibodies as surrogate markers and we feel that further work, measuring mycobacterial antigen or antibody in patient samples, is justified.

8.4 Conclusion

This study may be the first extensive investigation of protein expression by genetically characterised *M. tuberculosis* strains. The results described here have shown that protein expression by *M. tuberculosis* is dynamic and that genotypically different strains express proteins differentially. These differentially expressed proteins may influence strain "fitness", and may partially explain the observation of different frequencies of strain families within the study community. In addition, these findings have potential implications for future diagnostic and vaccine development initiatives. Differential protein expression may possibly explain why serodiagnosis of TB has been unsuccessful to date, and why previous *M. tuberculosis* infection is not able to adequately protect against subsequent infection.

Our findings confirm the extensive humoral heterogeneity between TB patients and support the development of immunodominant multi-antigen serodiagnostic tests and vaccines

since formulations based on single antigens may be of limited use in high incidence communities where patients are infected with a variety of genetically and phenotypically distinct strains. This study has emphasized the importance *M. tuberculosis* strain variation in TB pathogenesis, and careful analysis of the differences observed in this study may identify the functional significance of strain variation and ultimately the discovery of novel mechanisms of immune evasion strategies, strain "fitness", diagnostic and vaccine design strategies.

REFERENCES

A

Abulimiti, A., X. Qiu, J. Chen, Y. Liu, and Z. Chang. 2003. Reversible methionine sulfoxidation of *Mycobacterium tuberculosis* small heat shock protein Hsp16.3 and its possible role in scavenging oxidants. Biochem.Biophys.Res.Commun. **305**:87-93.

Agrewala, J. N. and R. J. Wilkinson. 1999. Influence of HLA-DR on the phenotype of CD4+ T lymphocytes specific for an epitope of the 16-kDa alpha-crystallin antigen of *Mycobacterium tuberculosis*. Eur.J.Immunol. **29**:1753-1761.

Al Zahrani, K., H. Al Jahdali, L. Poirier, P. Rene, M. L. Gennaro, and D. Menzies. 2000. Accuracy and utility of commercially available amplification and serologic tests for the diagnosis of minimal pulmonary tuberculosis. Am.J.Respir.Crit Care Med. **162**:1323-1329.

Amicosante, M., M. Houde, G. Guaraldi, and C. Saltini. 1999. Sensitivity and specificity of a multi-antigen ELISA test for the serological diagnosis of tuberculosis. Int.J.Tuberc.Lung Dis. **3**:736-740.

Andersen, A. B., P. Andersen, and L. Ljungqvist. 1992. Structure and function of a 40,000-molecular-weight protein antigen of *Mycobacterium tuberculosis*. Infect.Immun. **60**:2317-2323.

Andersen, P., D. Askgaard, L. Ljungqvist, J. Bennedsen, and I. Heron. 1991. Proteins released from *Mycobacterium tuberculosis* during growth. Infect.Immun. **59**:1905-1910.

Andersen, P. 1994. Effective vaccination of mice against *Mycobacterium tuberculosis* infection with a soluble mixture of secreted mycobacterial proteins. Infect.Immun. **62**:2536-2544.

Andersen, P., M. E. Munk, J. M. Pollock, and T. M. Doherty. 2000. Specific immune-based diagnosis of tuberculosis. Lancet **356**:1099-1104.

Andersen, P. 2001. TB vaccines: progress and problems. Trends Immunol. **22**:160-168.

Arias-Bouda, L. M., S. Kuijper, W. A. Van der, L. N. Nguyen, H. M. Jansen, and A. H. Kolk. 2003. Changes in avidity and level of immunoglobulin G antibodies to *Mycobacterium tuberculosis* in sera of patients undergoing treatment for pulmonary tuberculosis. Clin.Diagn.Lab Immunol. **10**:702-709.

B

Banu, S., N. Honore, B. Saint-Joanis, D. Philpott, M. C. Prevost, and S. T. Cole. 2002. Are the PE-PGRS proteins of *Mycobacterium tuberculosis* variable surface antigens? Mol.Microbiol. **44**:9-19.

Bao, L., W. Chen, H. Zhang, and X. Wang. 2003. Virulence, immunogenicity, and protective efficacy of two recombinant *Mycobacterium bovis* bacillus Calmette-Guérin strains expressing the antigen ESAT-6 from *Mycobacterium tuberculosis*. Infect.Immun. **71**:1656-1661.

Beggs, M. L., K. D. Eisenach, and M. D. Cave. 2000. Mapping of IS*6110* insertion sites in two epidemic strains of *Mycobacterium tuberculosis*. J.Clin.Microbiol. **38**:2923-2928.

Behr, M. A. and P. M. Small. 1997. Has BCG attenuated to impotence? Nature **389**:133-134.

Behr, M. A., S. A. Warren, H. Salamon, P. C. Hopewell, D. L. Ponce, C. L. Daley, and P. M. Small. 1999a. Transmission of *Mycobacterium tuberculosis* from patients smear-negative for acid-fast bacilli. Lancet **353**:444-449.

Behr, M. A., M. A. Wilson, W. P. Gill, H. Salamon, G. K. Schoolnik, S. Rane, and P. M. Small. 1999b. Comparative genomics of BCG vaccines by whole-genome DNA microarray. Science **284**:1520-1523.

Bellamy, R., C. Ruwende, T. Corrah, K. P. McAdam, H. C. Whittle, and A. V. Hill. 1998a. Variations in the NRAMP1 gene and susceptibility to tuberculosis in West Africans. N.Engl.J.Med. **338**:640-644.

Bellamy, R. J. and A. V. Hill. 1998b. Host genetic susceptibility to human tuberculosis. Novartis Found.Symp. **217**:3-13.

Bellamy, R., C. Ruwende, T. Corrah, K. P. McAdam, M. Thursz, H. C. Whittle, and A. V. Hill. 1999. Tuberculosis and chronic hepatitis B virus infection in Africans and variation in the vitamin D receptor gene. J.Infect.Dis. **179**:721-724.

Bentley-Hibbert, S. I., X. Quan, T. Newman, K. Huygen, and H. P. Godfrey. 1999. Pathophysiology of antigen 85 in patients with active tuberculosis: antigen 85 circulates as complexes with fibronectin and immunoglobulin G. Infect.Immun. **67**:581-588.

Bergmann, J. S. and G. L. Woods. 1996. Clinical evaluation of the Roche AMPLICOR PCR *Mycobacterium tuberculosis* test for detection of *M. tuberculosis* in respiratory specimens. J.Clin.Microbiol. **34**:1083-1085.

Bergmann, J. S., G. Yuoh, G. Fish, and G. L. Woods. 1999. Clinical evaluation of the enhanced Gen-Probe Amplified *Mycobacterium tuberculosis* Direct Test for rapid diagnosis of tuberculosis in prison inmates. J.Clin.Microbiol. **37**:1419-1425.

Betts, J. C., P. Dodson, S. Quan, A. P. Lewis, P. J. Thomas, K. Duncan, and R. A. McAdam. 2000. Comparison of the proteome of *Mycobacterium tuberculosis* strain H37Rv with clinical isolate CDC 1551. Microbiology **146**:3205-3216.

Betts, J. C. and M. A. Smith. 2001. Proteomics., p. 315-334. *In* T. Parish and N. G. Stoker (ed.), *Mycobacterium tuberculosis* Protocols, Methods in Microbiology, vol. 54. Humana Press, Totowa, NJ.

Betts, J. C., P. T. Lukey, L. C. Robb, R. A. McAdam, and K. Duncan. 2002a. Evaluation of a nutrient starvation model of *Mycobacterium tuberculosis* persistence by gene and protein expression profiling. Mol.Microbiol. **43**:717-731.

Betts, J. C. 2002b. Transcriptomics and proteomics: tools for the identification of novel drug targets and vaccine candidates for tuberculosis. IUBMB.Life **53**:239-242.

Beyers, N., R. P. Gie, H. L. Zietsman, M. Kunneke, J. Hauman, M. Tatley, and P. R. Donald. 1996. The use of a geographical information system (GIS) to evaluate the distribution of tuberculosis in a high-incidence community. S.Afr.Med.J. **86**:40-44.

Bhatnagar, R., A. N. Malaviya, S. Narayanan, P. Rajgopalan, R. Kumar, and O. P. Bharadwaj. 1977. Spectrum of immune response abnormalities in different clinical forms of tuberculosis. Am.Rev.Respir.Dis. **115**:207-212.

Bhattacharya, A., S. N. Ranadive, M. Kale, and S. Bhattacharya. 1986. Antibody-based enzyme-linked immunosorbent assay for determination of immune complexes in clinical tuberculosis. Am.Rev.Respir.Dis. **134**:205-209.

Bifani, P. J., B. Mathema, Z. Liu, S. L. Moghazeh, B. Shopsin, B. Tempalski, J. Driscol, R. Frothingham, J. M. Musser, P. Alcabes, and B. N. Kreiswirth. 1999. Identification of a W variant outbreak of *Mycobacterium tuberculosis* via population-based molecular epidemiology. JAMA **282**:2321-2327.

Bifani, P. J., B. Mathema, N. E. Kurepina, and B. N. Kreiswirth. 2002. Global dissemination of the *Mycobacterium tuberculosis* W-Beijing family strains. Trends Microbiol. **10**:45-52.

Bishai, W. R., A. M. Dannenberg, Jr., N. Parrish, R. Ruiz, P. Chen, B. C. Zook, W. Johnson, J. W. Boles, and M. L. Pitt. 1999. Virulence of *Mycobacterium tuberculosis* CDC1551 and H37Rv in rabbits evaluated by Lurie's pulmonary tubercle count method. Infect.Immun. **67**:4931-4934.

Bjellqvist, B., C. Pasquali, F. Ravier, J. C. Sanchez, and D. Hochstrasser. 1993. A nonlinear wide-range immobilized pH gradient for two-dimensional electrophoresis and its definition in a relevant pH scale. Electrophoresis **14**:1357-1365.

Blackstock, W. P. and M. P. Weir. 1999. Proteomics: quantitative and physical mapping of cellular proteins. Trends Biotechnol. **17**:121-127.

Bloom, B. R. and C. J. Murray. 1992. Tuberculosis: commentary on a reemergent killer. Science **257**:1055-1064.

Boon, C., R. Li, R. Qi, and T. Dick. 2001. Proteins of *Mycobacterium bovis* BCG induced in the Wayne dormancy model. J.Bacteriol. **183**:2672-2676.

Bosio, C. M., D. Gardner, and K. L. Elkins. 2000. Infection of B cell-deficient mice with CDC 1551, a clinical isolate of *Mycobacterium tuberculosis*: delay in dissemination and development of lung pathology. J.Immunol. **164**:6417-6425.

Bothamley, G. H., J. S. Beck, G. M. Schreuder, J. D'Amaro, R. R. de Vries, T. Kardjito, and J. Ivanyi. 1989. Association of tuberculosis and *M. tuberculosis*-specific antibody levels with HLA. J.Infect.Dis. **159**:549-555.

Bothamley, G. H., R. Rudd, F. Festenstein, and J. Ivanyi. 1992a. Clinical value of the measurement of *Mycobacterium tuberculosis* specific antibody in pulmonary tuberculosis. Thorax **47**:270-275.

Bothamley, G. H., J. S. Beck, R. C. Potts, J. M. Grange, T. Kardjito, and J. Ivanyi. 1992b. Specificity of antibodies and tuberculin response after occupational exposure to tuberculosis. J.Infect.Dis. **166**:182-186.

Bothamley, G. H. and R. M. Rudd. 1994. Clinical evaluation of a serological assay using a monoclonal antibody (TB72) to the 38 kDa antigen of *Mycobacterium tuberculosis*. Eur.Respir.J. **7**:240-246.
Bothamley, G. H. 1995. Serological diagnosis of tuberculosis. Eur.Respir.J.Suppl **20**:676s-688s.
Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal.Biochem. **72**:248-254.
Brandt, L., M. Elhay, I. Rosenkrands, E. B. Lindblad, and P. Andersen. 2000. ESAT-6 subunit vaccination against *Mycobacterium tuberculosis*. Infect.Immun. **68**:791-795.
Brandt, L., C. J. Feino, O. A. Weinreich, B. Chilima, P. Hirsch, R. Appelberg, and P. Andersen. 2002. Failure of the *Mycobacterium bovis* BCG vaccine: some species of environmental mycobacteria block multiplication of BCG and induction of protective immunity to tuberculosis. Infect.Immun. **70**:672-678.
Brennan, M. J., G. Delogu, Y. Chen, S. Bardarov, J. Kriakov, M. Alavi, and W. R. Jacobs, Jr. 2001. Evidence that mycobacterial PE_PGRS proteins are cell surface constituents that influence interactions with other cells. Infect.Immun. **69**:7326-7333.
Brennan, M. J. and U. Fruth. 2001. Global Forum on TB Vaccine Research and Development. World Health Organization, June 7-8 2001, Geneva. Tuberculosis.(Edinb.) **81**:365-368.
Brooks, J. V., A. A. Frank, M. A. Keen, J. T. Bellisle, and I. M. Orme. 2001. Boosting vaccine for tuberculosis. Infect.Immun. **69**:2714-2717.
Brosch, R., A. S. Pym, S. V. Gordon, and S. T. Cole. 2001. The evolution of mycobacterial pathogenicity: clues from comparative genomics. Trends Microbiol. **9**:452-458.
Burnette, W. N. 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal.Biochem. **112**:195-203.
Burridge, K. 1976. Changes in cellular glycoproteins after transformation: identification of specific glycoproteins and antigens in sodium dodecyl sulfate gels. Proc.Natl.Acad.Sci.U.S.A **73**:4457-4461.

C

Cahill, D. J. 2001. Protein and antibody arrays and their medical applications. J.Immunol.Methods **50**:81-91.
Camacho, L. R., D. Ensergueix, E. Perez, B. Gicquel, and C. Guilhot. 1999. Identification of a virulence gene cluster of *Mycobacterium tuberculosis* by signature-tagged transposon mutagenesis. Mol.Microbiol. **34**:257-267.
Campa, M., G. Benedettini, and P. Marelli. 1986. B and T lymphocytes regulated by idiotype anti-idiotype interactions inhibit delayed-type hypersensitivity to BCG in mice. Cell Immunol. **98**:93-103.
Cantarero, L. A., J. E. Butler, and J. W. Osborne. 1980. The adsorptive characteristics of proteins for polystyrene and their significance in solid-phase immunoassays. Anal.Biochem. **105**:375-382.
Cappelli, G., P. Volpe, A. Sanduzzi, A. Sacchi, V. Colizzi, and F. Mariani. 2001. Human macrophage gamma interferon decreases gene expression but not replication of *Mycobacterium tuberculosis*: analysis of the host-pathogen reciprocal influence on transcription in a comparison of strains H37Rv and CMT97. Infect.Immun. **69**:7262-7270.
Chambers, M. A., A. Williams, D. Gavier-Widen, A. Whelan, G. Hall, P. D. Marsh, B. R. Bloom, W. R. Jacobs, and R. G. Hewinson. 2000. Identification of a *Mycobacterium bovis* BCG auxotrophic mutant that protects guinea pigs against *M. bovis* and hematogenous spread of *Mycobacterium tuberculosis* without sensitization to tuberculin. Infect.Immun. **68**:7094-7099.
Chan, E. D., L. Heifets, and M. D. Iseman. 2000. Immunologic diagnosis of tuberculosis: a review. Tuber.Lung Dis. **80**:131-140.
Chan, J., X. D. Fan, S. W. Hunter, P. J. Brennan, and B. R. Bloom. 1991. Lipoarabinomannan, a possible virulence factor involved in persistence of *Mycobacterium tuberculosis* within macrophages. Infect.Immun. **59**:1755-1761.
Chandramuki, A., K. Lyashchenko, H. B. Kumari, N. Khanna, P. Brusasca, M. Gourie-Devi, P. Satishchandra, S. K. Shankar, V. Ravi, P. Alcabes, G. V. Kanaujia, and M. L. Gennaro. 2002. Detection of antibody to *Mycobacterium tuberculosis* protein antigens in the cerebrospinal fluid of patients with tuberculous meningitis. J.Infect.Dis. **186**:678-683.

Chaves, F., Z. Yang, H. el Hajj, M. Alonso, W. J. Burman, K. D. Eisenach, F. Dronda, J. H. Bates, and M. D. Cave. 1996. Usefulness of the secondary probe pTBN12 in DNA fingerprinting of *Mycobacterium tuberculosis*. J.Clin.Microbiol. **34**:1118-1123.
Chew, W. K., R. M. Lasaitis, F. A. Schio, and G. L. Gilbert. 1998. Clinical evaluation of the Mycobacteria Growth Indicator Tube (MGIT) compared with radiometric (Bactec) and solid media for isolation of *Mycobacterium* species. J.Med.Microbiol. **47**:821-827.
Choudhary, R. K., S. Mukhopadhyay, P. Chakhaiyar, N. Sharma, K. J. Murthy, V. M. Katoch, and S. E. Hasnain. 2003. PPE antigen Rv2430c of *Mycobacterium tuberculosis* induces a strong B-cell response. Infect.Immun. **71**:6338-6343.
Colangeli, R., J. S. Spencer, P. Bifani, A. Williams, K. Lyashchenko, M. A. Keen, P. J. Hill, J. Belisle, and M. L. Gennaro. 2000. MTSA-10, the product of the Rv3874 gene of *Mycobacterium tuberculosis*, elicits tuberculosis-specific, delayed-type hypersensitivity in guinea pigs. Infect.Immun. **68**:990-993.
Cole, S. T., R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S. V. Gordon, K. Eiglmeier, S. Gas, C. E. Barry, III, F. Tekaia, K. Badcock, D. Basham, D. Brown, T. Chillingworth, R. Connor, R. Davies, K. Devlin, T. Feltwell, S. Gentles, N. Hamlin, S. Holroyd, T. Hornsby, K. Jagels, and B. G. Barrell. 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature **393**:537-544.
Colebunders, R. and I. Bastian. 2000. A review of the diagnosis and treatment of smear-negative pulmonary tuberculosis. Int.J.Tuberc.Lung Dis. **4**:97-107.
Comstock, G. W. 1978. Tuberculosis in twins: a re-analysis of the Prophit survey. Am.Rev.Respir.Dis. **117**:621-624.
Constant, S. L. and K. Bottomly. 1997. Induction of Th1 and Th2 CD4+ T cell responses: the alternative approaches. Annu.Rev.Immunol. **15**:297-322.
Cooper, A. M., D. K. Dalton, T. A. Stewart, J. P. Griffin, D. G. Russell, and I. M. Orme. 1993. Disseminated tuberculosis in interferon gamma gene-disrupted mice. J.Exp.Med. **178**:2243-2247.
Cooper, A. M., J. Magram, J. Ferrante, and I. M. Orme. 1997. Interleukin 12 (IL-12) is crucial to the development of protective immunity in mice intravenously infected with *Mycobacterium tuberculosis*. J.Exp.Med. **186**:39-45.
Corbett, E. L., C. J. Watt, N. Walker, D. Maher, B. G. Williams, M. C. Raviglione, and C. Dye. 2003. The growing burden of tuberculosis: global trends and interactions with the HIV epidemic. Arch.Intern.Med. **163**:1009-1021.
Covert, B. A., J. S. Spencer, I. M. Orme, and J. T. Belisle. 2001. The application of proteomics in defining the T cell antigens of *Mycobacterium tuberculosis*. Proteomics. **1**:574-586.
Cox, J. S., B. Chen, M. McNeil, and W. R. Jacobs, Jr. 1999. Complex lipid determines tissue-specific replication of *Mycobacterium tuberculosis* in mice. Nature **402**:79-83.
Cruciani, M., M. Malena, O. Bosco, G. Gatti, and G. Serpelloni. 2001. The impact of human immunodeficiency virus type 1 on infectiousness of tuberculosis: a meta-analysis. Clin.Infect.Dis. **33**:1922-1930.

D

Daffe, M. 2000. The mycobacterial antigens 85 complex - from structure to function and beyond. Trends Microbiol. **8** :438-440.
Daffé, M. and P. Draper. 1998. The envelope layers of Mycobacteria with reference to their pathogenicity. Advances in Microbial Physiology **39**:131-203.
Daley, C. L., P. M. Small, G. F. Schecter, G. K. Schoolnik, R. A. McAdam, W. R. Jacobs, Jr., and P. C. Hopewell. 1992. An outbreak of tuberculosis with accelerated progression among persons infected with the human immunodeficiency virus. An analysis using restriction-fragment-length polymorphisms. N.Engl.J.Med. **326**:231-235.
Daniel, T. M. and B. W. Janicki. 1978. Mycobacterial antigens: a review of their isolation, chemistry, and immunological properties. Microbiol.Rev. **42**:84-113.
Daniel, T. M., M. J. Oxtoby, E. Pinto, and E. Moreno. 1981. The immune spectrum in patients with pulmonary tuberculosis. Am.Rev.Respir.Dis. **123**:556-559.
Daniel, T. M. and S. M. Debanne. 1987. The serodiagnosis of tuberculosis and other mycobacterial diseases by enzyme-linked immunosorbent assay. Am.Rev.Respir.Dis. **135**:1137-1151.

Dannenberg, A. M. Jr. and G. A. W. Rook. 1994. Pathogenesis of pulmonary tuberculosis: an interplay of tissue-damaging and macrophage-activating immune responses-dual mechanisms that control bacillary multiplication, p. 459-483. *In* B. R. Bloom (ed.), Tuberculosis: Pathogenesis, Protection, and Control. American Society for Microbiology, Washington, DC.
De Cock, K. M., B. Soro, I. M. Coulibaly, and S. B. Lucas. 1992. Tuberculosis and HIV infection in sub-Saharan Africa. JAMA **268**:1581-1587.
Delcher, A. L., S. Kasif, R. D. Fleischmann, J. Peterson, O. White, and S. L. Salzberg. 1999. Alignment of whole genomes. Nucleic Acids Res. **27**:2369-2376.
Delogu, G., A. Howard, F. M. Collins, and S. L. Morris. 2000. DNA vaccination against tuberculosis: expression of a ubiquitin-conjugated tuberculosis protein enhances antimycobacterial immunity. Infect.Immun. **68**:3097-3102.
Deretic, V. and R. A. Fratti. 1999. *Mycobacterium tuberculosis* phagosome. Mol.Microbiol. **31**:1603-1609.
Desjardin, L. E., M. D. Perkins, K. Wolski, S. Haun, L. Teixeira, Y. Chen, J. L. Johnson, J. J. Ellner, R. Dietze, J. Bates, M. D. Cave, and K. D. Eisenach. 1999. Measurement of sputum *Mycobacterium tuberculosis* messenger RNA as a surrogate for response to chemotherapy. Am.J.Respir.Crit Care Med. **160**:203-210.
Desjardin, L. E., L. G. Hayes, C. D. Sohaskey, L. G. Wayne, and K. D. Eisenach. 2001. Microaerophilic induction of the alpha-crystallin chaperone protein homologue (hspX) mRNA of *Mycobacterium tuberculosis*. J.Bacteriol. **183**:5311-5316.
Döffinger, R., S. Dupuis, C. Picard, C. Fieschi, J. Feinberg, G. Barcenas-Morales, and J. L. Casanova. 2002. Inherited disorders of IL-12- and IFNgamma-mediated immunity: a molecular genetics update. Mol.Immunol. **38**:903-909.
Doherty, T. M., A. Demissie, J. Olobo, D. Wolday, S. Britton, T. Eguale, P. Ravn, and P. Andersen. 2002. Immune responses to the *Mycobacterium tuberculosis*-specific antigen ESAT-6 signal subclinical infection among contacts of tuberculosis patients. J.Clin.Microbiol. **40**:704-706.
Drowart, A., K. Huygen, J. De Bruyn, J. C. Yernault, C. M. Farber, and J. P. Van Vooren. 1991. Antibody levels to whole culture filtrate antigens and to purified P32 during treatment of smear-positive tuberculosis. Chest **100**:685-687.
du Plessis, D. G., R. Warren, M. Richardson, J. J. Joubert, and P. D. van Helden. 2001. Demonstration of reinfection and reactivation in HIV-negative autopsied cases of secondary tuberculosis: multilesional genotyping of *Mycobacterium tuberculosis* utilizing IS*6110* and other repetitive element-based DNA fingerprinting. Tuberculosis.(Edinb.) **81**:211-220.
Dubos, R. and J. Dubos. 1987. The white plague, p. 28-43. Rutgers University Press, New Brunswick and London.
Duncan, K. 2003. Progress in TB drug development and what is still needed. Tuberculosis.(Edinb.) **83**:201-207.
Dye, C., S. Scheele, P. Dolin, V. Pathania, and M. C. Raviglione. 1999. Consensus statement. Global burden of tuberculosis: estimated incidence, prevalence, and mortality by country. WHO Global Surveillance and Monitoring Project. JAMA **282**:677-686.

E

Enarson, D. A. and A. Rouillon. 1994. The epidemiological basis of tuberculosis control. p. 19-32. *In* P. D. O. Davies (ed.), Clinical Tuberculosis. Chapman and Hall, London.
Engers, H. D., V. Houba, J. Bennedsen, T. M. Buchanan, S. D. Chaparas, G. Kadival, O. Closs, J. R. David, J. D. A. van Embden, T. Godal, S. A. Mustafa, J. Ivanyi, D. B. Young, S. H. E. Kaufmann, A. G. Khomenko, A. H. J. Kolk, M. Kubin, J. A. Louis, P. Minden, T. M. Shinnick, L. Trnka, and R. A. Young. 1986. Results of a World Health Organization-Sponsored Workshop to Characterize Antigens Recognized by *Mycobacterium*-Specific monoclonal Antibodies. Infect.Immun. **51**:718-720.
Engvall, E. and P. Perlman. 1971. Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. Immunochemistry. **8**:871-874.
Epstein, M. D., N. W. Schluger, A. L. Davidow, S. Bonk, W. N. Rom, and B. Hanna. 1998. Time to detection of *Mycobacterium tuberculosis* in sputum culture correlates with outcome in patients receiving treatment for pulmonary tuberculosis. Chest **113**:379-386.
Ernst, J. D. 1998. Macrophage receptors for *Mycobacterium tuberculosis*. Infect.Immun. **66**:1277-1281.

Espitia, C., J. P. Laclette, M. Mondragon-Palomino, A. Amador, J. Campuzano, A. Martens, M. Singh, R. Cicero, Y. Zhang, and C. Moreno. 1999. The PE-PGRS glycine-rich proteins of *Mycobacterium tuberculosis*: a new family of fibronectin-binding proteins? Microbiology **145** :3487-3495.

F

Falero-Diaz, G., S. Challacombe, D. Banerjee, G. Douce, A. Boyd, and J. Ivanyi. 2000. Intranasal vaccination of mice against infection with *Mycobacterium tuberculosis*. Vaccine **18**:3223-3229.
Feng, C. G., U. Palendira, C. Demangel, J. M. Spratt, A. S. Malin, and W. J. Britton. 2001. Priming by DNA immunization augments protective efficacy of *Mycobacterium bovis* Bacille Calmette-Guérin against tuberculosis. Infect.Immun. **69**:4174-4176.
Fenhalls, G., L. Stevens, L. Moses, J. Bezuidenhout, J. C. Betts, P. P. Helden, P. T. Lukey, and K. Duncan. 2002. In situ detection of *Mycobacterium tuberculosis* transcripts in human lung granulomas reveals differential gene expression in necrotic lesions. Infect.Immun. **70**:6330-6338.
Fenton, M. J. and M. W. Vermeulen. 1996. Immunopathology of tuberculosis: roles of macrophages and monocytes. Infect.Immun. **64**:683-690.
Fine, P. E. 1989. The BCG story: lessons from the past and implications for the future. Rev.Infect.Dis. **11 Suppl 2** :S353-S359.
Fine, P. E., J. A. Sterne, J. M. Ponnighaus, and R. J. Rees. 1994. Delayed-type hypersensitivity, mycobacterial vaccines and protective immunity. Lancet **344**:1245-1249.
Fine, P. E. 1995. Variation in protection by BCG: implications of and for heterologous immunity. Lancet **346**:1339-1345.
Fleischmann, R. D., D. Alland, J. A. Eisen, L. Carpenter, O. White, J. Peterson, R. DeBoy, R. Dodson, M. Gwinn, D. Haft, E. Hickey, J. F. Kolonay, W. C. Nelson, L. A. Umayam, M. Ermolaeva, S. L. Salzberg, A. Delcher, T. Utterback, J. Weidman, H. Khouri, J. Gill, A. Mikula, W. Bishai, J. W. Jacobs, Jr., J. C. Venter, and C. M. Fraser. 2002. Whole-genome comparison of *Mycobacterium tuberculosis* clinical and laboratory strains. J.Bacteriol. **184**:5479-5490.
Flesch, I. and S. H. Kaufmann. 1987. Mycobacterial growth inhibition by interferon-gamma-activated bone marrow macrophages and differential susceptibility among strains of *Mycobacterium tuberculosis*. J.Immunol. **138**:4408-4413.
Flesch, I. E. and S. H. Kaufmann. 1990. Activation of tuberculostatic macrophage functions by gamma interferon, interleukin-4, and tumor necrosis factor. Infect.Immun. **58**:2675-2677.
Flores, J. and C. Espitia. 2003. Differential expression of PE and PE_PGRS genes in *Mycobacterium tuberculosis* strains. Gene **318**:75-81.
Flynn, J. L., J. Chan, K. J. Triebold, D. K. Dalton, T. A. Stewart, and B. R. Bloom. 1993. An essential role for interferon gamma in resistance to *Mycobacterium tuberculosis* infection. J.Exp.Med. **178**:2249-2254.
Flynn, J. L., M. M. Goldstein, J. Chan, K. J. Triebold, K. Pfeffer, C. J. Lowenstein, R. Schreiber, T. W. Mak, and B. R. Bloom. 1995. Tumor necrosis factor-alpha is required in the protective immune response against *Mycobacterium tuberculosis* in mice. Immunity. **2**:561-572.
Flynn, J. L. and J. Chan. 2001. Immunology of tuberculosis. Annu.Rev.Immunol. **19**:93-129.
Friedman, C. R., G. C. Quinn, B. N. Kreiswirth, D. C. Perlman, N. Salomon, N. Schluger, M. Lutfey, J. Berger, N. Poltoratskaia, and L. W. Riley. 1997. Widespread dissemination of a drug-susceptible strain of *Mycobacterium tuberculosis*. J.Infect.Dis. **176**:478-484.
Frucht, D. M. and S. M. Holland. 1996. Defective monocyte costimulation for IFN-gamma production in familial disseminated *Mycobacterium avium* complex infection: abnormal IL-12 regulation. J.Immunol. **157**:411-416.

G

Gamboa, F., G. Fernandez, E. Padilla, J. M. Manterola, J. Lonca, P. J. Cardona, L. Matas, and V. Ausina. 1998. Comparative evaluation of initial and new versions of the Gen-Probe Amplified *Mycobacterium tuberculosis* Direct Test for direct detection of *Mycobacterium tuberculosis* in respiratory and nonrespiratory specimens. J.Clin.Microbiol. **36**:684-689.

Garbe, T. R., N. S. Hibler, and V. Deretic. 1996. Isoniazid induces expression of the antigen 85 complex in *Mycobacterium tuberculosis*. Antimicrob.Agents Chemother. **40**:1754-1756.
Gey Van Pittius, N. C., J. Gamieldien, W. Hide, G. D. Brown, R. J. Siezen, and A. D. Beyers. 2001. The ESAT-6 gene cluster of *Mycobacterium tuberculosis* and other high G+C Gram-positive bacteria. Genome Biol. **2**:RESEARCH0044.
Gibson, J. A., J. M. Grange, J. S. Beck, and T. Kardjito. 1987. Specific antibody in the subclasses of immunoglobulin G in patients with smear-positive pulmonary tuberculosis. Eur.J.Respir.Dis. **70**:29-34.
Glatman-Freedman, A. and A. Casadevall. 1998. Serum therapy for tuberculosis revisited: reappraisal of the role of antibody-mediated immunity against *Mycobacterium tuberculosis*. Clin.Microbiol.Rev. **11**:514-532.
Glatman-Freedman, A., A. J. Mednick, N. Lendvai, and A. Casadevall. 2000. Clearance and organ distribution of *Mycobacterium tuberculosis* lipoarabinomannan (LAM) in the presence and absence of LAM-binding immunoglobulin M. Infect.Immun. **68**:335-341.
Glynn, J. R., J. Whiteley, P. J. Bifani, K. Kremer, and D. van Soolingen. 2002. Worldwide occurrence of Beijing/W strains of *Mycobacterium tuberculosis*: a systematic review. Emerg.Infect.Dis. **8**:843-849.
Goletti, D., D. Weissman, R. W. Jackson, N. M. Graham, D. Vlahov, R. S. Klein, S. S. Munsiff, L. Ortona, R. Cauda, and A. S. Fauci. 1996. Effect of *Mycobacterium tuberculosis* on HIV replication. Role of immune activation. J.Immunol. **157**:1271-1278.
Gonzalez-Juarrero, M., O. C. Turner, J. Turner, P. Marietta, J. V. Brooks, and I. M. Orme. 2001. Temporal and spatial arrangement of lymphocytes within lung granulomas induced by aerosol infection with *Mycobacterium tuberculosis*. Infect.Immun. **69**:1722-1728.
Gorg, A., C. Obermaier, G. Boguth, A. Harder, B. Scheibe, R. Wildgruber, and W. Weiss. 2000. The current state of two-dimensional electrophoresis with immobilized pH gradients. Electrophoresis **21**:1037-1053.
Groenen, P. M., A. E. Bunschoten, D. van Soolingen, and J. D. van Embden. 1993. Nature of DNA polymorphism in the direct repeat cluster of *Mycobacterium tuberculosis*; application for strain differentiation by a novel typing method. Mol.Microbiol. **10**:1057-1065.
Guleria, I., R. Teitelbaum, R. A. McAdam, G. Kalpana, W. R. Jacobs, Jr., and B. R. Bloom. 1996. Auxotrophic vaccines for tuberculosis. Nat.Med. **2**:334-337.
Gupta, S., R. Bhatia, and K. K. Datta. 1997. Serological diagnosis of childhood tuberculosis by estimation of mycobacterial antigen 60-specific immunoglobulins in the serum. Tuber.Lung Dis. **78**:21-27.
Gygi, S. P., B. Rist, and R. Aebersold. 2000. Measuring gene expression by quantitative proteome analysis. Curr.Opin.Biotechnol. **11**:396-401.

H

Haga, S., R. Yamaguchi, S. Nagai, K. Matsuo, A. Yamazaki, and R. M. Nakamura. 1995. Delayed-type hypersensitivity to a recombinant mycobacterial antigen, MPB64, in guinea pigs sensitized to *Mycobacterium tuberculosis* or *Mycobacterium bovis* BCG. J.Leukoc.Biol. **57**:221-225.
Harboe, M. and H. G. Wiker. 1992. The 38-kDa protein of *Mycobacterium tuberculosis*: a review. J.Infect.Dis. **166**:874-884.
Harboe, M., T. Oettinger, H. G. Wiker, I. Rosenkrands, and P. Andersen. 1996. Evidence for occurrence of the ESAT-6 protein in *Mycobacterium tuberculosis* and virulent *Mycobacterium bovis* and for its absence in *Mycobacterium bovis* BCG. Infect.Immun. **64**:16-22.
Harth, G., B. Y. Lee, J. Wang, D. L. Clemens, and M. A. Horwitz. 1996. Novel insights into the genetics, biochemistry, and immunocytochemistry of the 30-kilodalton major extracellular protein of *Mycobacterium tuberculosis*. Infect.Immun. **64**:3038-3047.
Heifets, L. B. and R. C. Good. 1994. Current laboratory methods for the diagnosis of tuberculosis., p. 85-110. *In* B. R. Bloom (ed.), Tuberculosis: pathogenesis, protection, and control. American Society for Microbiology Press, Washington DC.
Hellyer, T. J., L. E. Desjardin, L. Teixeira, M. D. Perkins, M. D. Cave, and K. D. Eisenach. 1999. Detection of viable *Mycobacterium tuberculosis* by reverse transcriptase-strand displacement amplification of mRNA. J.Clin.Microbiol. **37**:518-523.

Hendrickson, R. C., J. F. Douglass, L. D. Reynolds, P. D. McNeill, D. Carter, S. G. Reed, and R. L. Houghton. 2000. Mass spectrometric identification of mtb81, a novel serological marker for tuberculosis. J.Clin.Microbiol. **38**:2354-2361.

Herbert, B. 1999. Advances in protein solubilisation for two-dimensional electrophoresis. Electrophoresis **20**:660-663.

Herbert, B. R., M. P. Molloy, A. A. Gooley, B. J. Walsh, W. G. Bryson, and K. L. Williams. 1998. Improved protein solubility in two-dimensional electrophoresis using tributyl phosphine as reducing agent. Electrophoresis **19**:845-851.

Hingley-Wilson, S. M., V. K. Sambandamurthy, and W. R. Jacobs, Jr. 2003. Survival perspectives from the world's most successful pathogen, *Mycobacterium tuberculosis*. Nat.Immunol. **4**:949-955.

Hoal-van Helden, E. G., D. Hon, L. A. Lewis, N. Beyers, and P. D. van Helden. 2001a. Mycobacterial growth in human macrophages: variation according to donor, inoculum and bacterial strain. Cell Biol.Int. **25**:71-81.

Hoal-van Helden, E. G., L. A. Stanton, R. Warren, M. Richardson, and P. D. van Helden. 2001b. Diversity of in vitro cytokine responses by human macrophages to infection by *Mycobacterium tuberculosis* strains. Cell Biol.Int. **25**:83-90.

Hondalus, M. K., S. Bardarov, R. Russell, J. Chan, W. R. Jacobs, Jr., and B. R. Bloom. 2000. Attenuation of and protection induced by a leucine auxotroph of *Mycobacterium tuberculosis*. Infect.Immun. **68**:2888-2898.

Hopewell, P. C. 1994. Overview of Clinical Tuberculosis, p. 25-46. *In* B. Bloom (ed.), Tuberculosis: Pathogenesis, Protection and Control. ASM Press, Washington DC.

Horwitz, M. A., B. W. Lee, B. J. Dillon, and G. Harth. 1995. Protective immunity against tuberculosis induced by vaccination with major extracellular proteins of *Mycobacterium tuberculosis*. Proc.Natl.Acad.Sci.U.S.A **92**:1530-1534.

Horwitz, M. A., G. Harth, B. J. Dillon, and S. Maslesa-Galic'. 2000. Recombinant bacillus Calmette-Guérin (BCG) vaccines expressing the *Mycobacterium tuberculosis* 30-kDa major secretory protein induce greater protective immunity against tuberculosis than conventional BCG vaccines in a highly susceptible animal model. Proc.Natl.Acad.Sci.U.S.A **97**:13853-13858.

Horwitz, M. A. and G. Harth. 2003. A new vaccine against tuberculosis affords greater survival after challenge than the current vaccine in the guinea pig model of pulmonary tuberculosis. Infect.Immun. **71**:1672-1679.

Houghton, R. L., M. J. Lodes, D. C. Dillon, L. D. Reynolds, C. H. Day, P. D. McNeill, R. C. Hendrickson, Y. A. Skeiky, D. P. Sampaio, R. Badaro, K. P. Lyashchenko, and S. G. Reed. 2002. Use of multiepitope polyproteins in serodiagnosis of active tuberculosis. Clin.Diagn.Lab Immunol. **9**:883-891.

Huang, T. S., C. S. Chen, S. S. Lee, W. K. Huang, and Y. C. Liu. 2001. Comparison of the BACTEC MGIT 960 and BACTEC 460TB systems for detection of mycobacteria in clinical specimens. Ann.Clin.Lab Sci. **31**:279-283.

Huebner, R. E., M. F. Schein, and J. B. Bass, Jr. 1993. The tuberculin skin test. Clin.Infect.Dis. **17**:968-975.

Hunter, S. W., H. Gaylord, and P. J. Brennan. 1986. Structure and antigenicity of the phosphorylated lipopolysaccharide antigens from the leprosy and tubercle bacilli. J.Biol.Chem. **261**:12345-12351.

Hussain, R., R. W. Poindexter, E. A. Ottesen, and C. B. Reimer. 1986a. Use of monoclonal antibodies to quantify subclasses of human IgG. II. Enzyme immunoassay to define antigen specific (anti-filarial) IgG subclass antibodies. J.Immunol.Methods **94**:73-80.

Hussain, R., R. W. Poindexter, R. Wistar, and C. B. Reimer . 1986b. Use of monoclonal antibodies to quantify subclasses of human IgG. I. Development of two-site immunoenzymometric assays for total IgG subclass determinations. J.Immunol.Methods **93**:89-96.

Hussain, R., G. Dawood, N. Abrar, Z. Toossi, A. Minai, M. Dojki, and J. J. Ellner. 1995. Selective increases in antibody isotypes and immunoglobulin G subclass responses to secreted antigens in tuberculosis patients and healthy household contacts of the patients. Clin.Diagn.Lab Immunol. **2**:726-732.

Hussain, R., H. Shiratsuchi, J. J. Ellner, and R. S. Wallis. 2000. PPD-specific IgG1 antibody subclass upregulate tumour necrosis factor expression in PPD-stimulated monocytes: possible link with disease pathogenesis in tuberculosis. Clin.Exp.Immunol. **119**:449-455.

Hussain, R., H. Shiratsuchi, M. Phillips, J. Ellner, and R. S. Wallis. 2001. Opsonizing antibodies (IgG1) up-regulate monocyte proinflammatory cytokines tumour necrosis factor-alpha (TNF-alpha) and IL-6 but

not anti-inflammatory cytokine IL-10 in mycobacterial antigen-stimulated monocytes-implications for pathogenesis. Clin.Exp.Immunol. **123**:210-218.
Hussain, R., A. Kaleem, F. Shahid, M. Dojki, B. Jamil, H. Mehmood, G. Dawood, and H. M. Dockrell. 2002. Cytokine profiles using whole-blood assays can discriminate between tuberculosis patients and healthy endemic controls in a BCG-vaccinated population. J.Immunol.Methods **264**:95-108.
Huygen, K. 1998. DNA vaccines: application to tuberculosis. Int.J.Tuberc.Lung Dis. **2**:971-978.
Hwang, P. H. and J. S. Kim. 1993. PPD-specific IgG and IgG subclasses in the sera of pulmonary tuberculosis patients. J.Korean Med.Sci. **8**:1-9.

I

Imaz, M. S. and E. Zerbini. 2000. Antibody response to culture filtrate antigens of *Mycobacterium tuberculosis* during and after treatment of tuberculosis patients. Int.J.Tuberc.Lung Dis. **4**:562-569.
Iseman, M. D. and J. A. Sbarbaro. 1991. Short-course chemotherapy of tuberculosis. Hail Britannia (and friends)! Am.Rev.Respir.Dis. **143**:697-698.

J

Jackson, M., S. W. Phalen, M. Lagranderie, D. Ensergueix, P. Chavarot, G. Marchal, D. N. McMurray, B. Gicquel, and C. Guilhot. 1999. Persistence and protective efficacy of a *Mycobacterium tuberculosis* auxotroph vaccine. Infect.Immun. **67**:2867-2873.
Jacobs, W., P. Brennan, G. Curlin, A. Ginsberg, M. Adams, R. Fleischmann, C. Fraser, J. C. Venter, T. Shinnick, W. Bishai, H. Smith, K. Stover, and G. Hatfull. 1996. Comparative sequencing. Science **274**:17-18.
Jung, Y. J., R. LaCourse, L. Ryan, and R. J. North. 2002. Evidence inconsistent with a negative influence of T helper 2 cells on protection afforded by a dominant T helper 1 response against *Mycobacterium tuberculosis* lung infection in mice. Infect.Immun. **70**:6436-6443.
Jungblut, P. R., U. E. Schaible, H. J. Mollenkopf, U. Zimny-Arndt, B. Raupach, J. Mattow, P. Halada, S. Lamer, K. Hagens, and S. H. Kaufmann. 1999. Comparative proteome analysis of *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG strains: towards functional genomics of microbial pathogens. Mol.Microbiol. **33**:1103-1117.
Jungblut, P. R., E. C. Muller, J. Mattow, and S. H. Kaufmann. 2001. Proteomics reveals open reading frames in *Mycobacterium tuberculosis* H37Rv not predicted by genomics. Infect.Immun. **69**:5905-5907.

K

Kamath, A. T., C. G. Feng, M. Macdonald, H. Briscoe, and W. J. Britton. 1999. Differential protective efficacy of DNA vaccines expressing secreted proteins of *Mycobacterium tuberculosis*. Infect.Immun. **67**:1702-1707.
Kardjito, T., I. Handoyo, and J. M. Grange. 1982. Diagnosis of active tuberculosis by immunological methods. 1. The effect of tuberculin reactivity and previous BCG vaccination on the antibody levels determined by ELISA. Tubercle. **63**:269-274.
Karonga Trial Prevention Group. 1996. Randomised controlled trial of single BCG, repeated BCG, or combined BCG and killed *Mycobacterium leprae* vaccine for prevention of leprosy and tuberculosis in Malawi. Lancet **348**:17-24.
Kato-Maeda, M., J. T. Rhee, T. R. Gingeras, H. Salamon, J. Drenkow, N. Smittipat, and P. M. Small. 2001. Comparing genomes within the species *Mycobacterium tuberculosis*. Genome Res. **11**:547-554.
Khanolkar-Young, S., A. H. J. Kolk, A. B. Andersen, J. Bennedsen, P. J. Brennan, B. Rivoire, S. Kuijper, K. P. W. J. McAdam, C. Abe, H. V. Batra, S. D. Chaparas, G. Damiani, M. Singh, and H. D. Engers. 1992. Results of the third immunology of leprosy/immunology of tuberculosis antimycobacterial monoclonal antibody workshop. Infect.Immun. **60**:3925-3927.
Koch, R. 1882. Die Aetiologie der Tuberculos. Ber.Klin.Wochenschr. **19**:21.

Kochi, A., B. Vareldzis, and K. Styblo. 1993. Multidrug-resistant tuberculosis and its control. Res.Microbiol. **144** :104-110.
Kwissa, M., E. B. Lindblad, R. Schirmbeck, and J. Reimann. 2003. Codelivery of a DNA vaccine and a protein vaccine with aluminum phosphate stimulates a potent and multivalent immune response. J.Mol.Med. **81**:502-510.
Kyhse-Andersen, J. 1984. Electroblotting of multiple gels: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. J.Biochem.Biophys.Methods **10**:203-209.

L

Laal, S., K. M. Samanich, M. G. Sonnenberg, S. Zolla-Pazner, J. M. Phadtare, and J. T. Belisle. 1997. Human humoral responses to antigens of *Mycobacterium tuberculosis*: immunodominance of high-molecular-mass antigens. Clin.Diagn.Lab Immunol. **4**:49-56.
Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**:680-685.
Lalvani, A., A. A. Pathan, H. McShane, R. J. Wilkinson, M. Latif, C. P. Conlon, G. Pasvol, and A. V. Hill. 2001. Rapid detection of *Mycobacterium tuberculosis* infection by enumeration of antigen-specific T cells. Am.J.Respir.Crit Care Med. **163**:824-828.
Landowski, C. P., H. P. Godfrey, S. I. Bentley-Hibbert, X. Liu, Z. Huang, R. Sepulveda, K. Huygen, M. L. Gennaro, F. H. Moy, S. A. Lesley, and M. Haak-Frendscho. 2001. Combinatorial use of antibodies to secreted mycobacterial proteins in a host immune system-independent test for tuberculosis. J.Clin.Microbiol. **39**:2418-2424.
Laux, D. L. 1991. Effects of luminol on the subsequent analysis of bloodstains. J.Forensic Sci. **36**:1512-1520.
Lee, B. Y., S. A. Hefta, and P. J. Brennan. 1992. Characterization of the major membrane protein of virulent *Mycobacterium tuberculosis*. Infect.Immun. **60**:2066-2074.
Lee, B. Y. and M. A. Horwitz. 1995. Identification of macrophage and stress-induced proteins of *Mycobacterium tuberculosis*. J.Clin.Invest **96**:245-249.
Lein, A. D., C. F. von Reyn, P. Ravn, C. R. Horsburgh, Jr., L. N. Alexander, and P. Andersen. 1999. Cellular immune responses to ESAT-6 discriminate between patients with pulmonary disease due to *Mycobacterium avium* complex and those with pulmonary disease due to *Mycobacterium tuberculosis*. Clin.Diagn.Lab Immunol. **6**:606-609.
Lenzini, L., P. Rottoli, and L. Rottoli. 1977. The spectrum of human tuberculosis. Clin.Exp.Immunol. **27**:230-237.
Levy, H., C. Feldman, H. Sacho, M. H. van der, J. Kallenbach, and H. Koornhof. 1989. A reevaluation of sputum microscopy and culture in the diagnosis of pulmonary tuberculosis. Chest **95**:1193-1197.
Lewis, K. N., R. Liao, K. M. Guinn, M. J. Hickey, S. Smith, M. A. Behr, and D. R. Sherman. 2003. Deletion of RD1 from *Mycobacterium tuberculosis* mimics bacille Calmette-Guérin attenuation. J.Infect.Dis. **187**:117-123.
Loebel, R. O., E. Shorr, and H. B. Richardson. 1933. The influence of adverse conditions upon the respiratory metabolism and growth of human tubercle bacilli. J.Bacteriol. **26**:167-200.
Lopez, B., D. Aguilar, H. Orozco, M. Burger, C. Espitia, V. Ritacco, L. Barrera, K. Kremer, R. Hernandez-Pando, K. Huygen, and D. van Soolingen. 2003. A marked difference in pathogenesis and immune response induced by different *Mycobacterium tuberculosis* genotypes. Clin.Exp.Immunol. **133**:30-37.
Lowrie, D. B., R. E. Tascon, V. L. Bonato, V. M. Lima, L. H. Faccioli, E. Stavropoulos, M. J. Colston, R. G. Hewinson, K. Moelling, and C. L. Silva. 1999. Therapy of tuberculosis in mice by DNA vaccination. Nature **400**:269-271.
Lundgren, M., U. Persson, P. Larsson, C. Magnusson, C. I. Smith, L. Hammarstrom, and E. Severinson. 1989. Interleukin 4 induces synthesis of IgE and IgG4 in human B cells. Eur.J.Immunol. **19**:1311-1315.
Lyashchenko, K., R. Colangeli, M. Houde, H. Al Jahdali, D. Menzies, and M. L. Gennaro. 1998a. Heterogeneous antibody responses in tuberculosis. Infect.Immun. **66**:3936-3940.
Lyashchenko, K. P., J. M. Pollock, R. Colangeli, and M. L. Gennaro. 1998b. Diversity of antigen recognition by serum antibodies in experimental bovine tuberculosis. Infect.Immun. **66**:5344-5349.

M

Manabe, Y. C., A. M. Dannenberg, Jr., S. K. Tyagi, C. L. Hatem, M. Yoder, S. C. Woolwine, B. C. Zook, M. L. Pitt, and W. R. Bishai. 2003. Different strains of *Mycobacterium tuberculosis* cause various spectrums of disease in the rabbit model of tuberculosis. Infect.Immun. **71** :6004-6011.

Manca, C., L. Tsenova, C. E. Barry, III, A. Bergtold, S. Freeman, P. A. Haslett, J. M. Musser, V. H. Freedman, and G. Kaplan. 1999. *Mycobacterium tuberculosis* CDC1551 induces a more vigorous host response *in vivo* and *in vitro*, but is not more virulent than other clinical isolates. J.Immunol. **162**:6740-6746.

Marchant, A., T. Goetghebuer, M. O. Ota, I. Wolfe, S. J. Ceesay, D. De Groote, T. Corrah, S. Bennett, J. Wheeler, K. Huygen, P. Aaby, K. P. McAdam, and M. J. Newport. 1999. Newborns develop a Th1-type immune response to *Mycobacterium bovis* bacillus Calmette-Guérin vaccination. J.Immunol. **163**:2249-2255.

Martin, E., A. T. Kamath, H. Briscoe, and W. J. Britton. 2003. The combination of plasmid interleukin-12 with a single DNA vaccine is more effective than *Mycobacterium bovis* (bacille Calmette-Guérin) in protecting against systemic *Mycobacterium avium* infection. Immunology **109**:308-314.

Mattow, J., P. R. Jungblut, U. E. Schaible, H. J. Mollenkopf, S. Lamer, U. Zimny-Arndt, K. Hagens, E. C. Muller, and S. H. Kaufmann. 2001. Identification of proteins from *Mycobacterium tuberculosis* missing in attenuated *Mycobacterium bovis* BCG strains. Electrophoresis **22**:2936-2946.

McShane, H., R. Brookes, S. C. Gilbert, and A. V. Hill. 2001. Enhanced immunogenicity of CD4(+) t-cell responses and protective efficacy of a DNA-modified vaccinia virus Ankara prime-boost vaccination regimen for murine tuberculosis. Infect.Immun. **69**:681-686.

Meyers, P. R., W. R. Bourn, L. M. Steyn, P. D. van Helden, A. D. Beyers, and G. D. Brown. 1998. Novel method for rapid measurement of growth of mycobacteria in detergent-free media. J.Clin.Microbiol. **36**:2752-2754.

Migliori, G. B., A. Borghesi, A. Spanevello, P. Eriki, M. Raviglione, G. Maciocco, A. Morandi, L. Ballardini, and M. Neri. 1994. Risk of infection and estimated incidence of tuberculosis in northern Uganda. Eur.Respir.J. **7**:946-953.

Mitchison, D. A., J. B. Selkon, and J. Lloyd. 1963. Virulence in the guinea-pig, susceptibility to hydrogen peroxide, and catalase activity of isoniazid-sensitive tubercle bacilli from South Indian and British patients. J.Pathol.Bacteriol. **86**:377-386.

Mitchison, D. A. 1993. Assessment of new sterilizing drugs for treating pulmonary tuberculosis by culture at 2 months. Am.Rev.Respir.Dis. **147**:1062-1063.

Modlin, R. L. 1994. Th1-Th2 paradigm: insights from leprosy. J.Invest Dermatol. **102**:828-832.

Monahan, I. M., J. Betts, D. K. Banerjee, and P. D. Butcher. 2001. Differential expression of mycobacterial proteins following phagocytosis by macrophages. Microbiology **147**:459-471.

Morris, S., C. Kelley, A. Howard, Z. Li, and F. Collins. 2000. The immunogenicity of single and combination DNA vaccines against tuberculosis. Vaccine **18**:2155-2163.

Murphy, E. E., G. Terres, S. E. Macatonia, C. S. Hsieh, J. Mattson, L. Lanier, M. Wysocka, G. Trinchieri, K. Murphy, and A. O'Garra. 1994. B7 and interleukin 12 cooperate for proliferation and interferon gamma production by mouse T helper clones that are unresponsive to B7 costimulation. J.Exp.Med. **180**:223-231.

Murray, P. J., A. Aldovini, and R. A. Young. 1996. Manipulation and potentiation of antimycobacterial immunity using recombinant bacille Calmette-Guérin strains that secrete cytokines. Proc.Natl.Acad.Sci.U.S.A **93**:934-939.

Musser, J. M., A. Amin, and S. Ramaswamy. 2000. Negligible genetic diversity of *Mycobacterium tuberculosis* host immune system protein targets: evidence of limited selective pressure. Genetics **155**:7-16.

N

North, R. J. and A. A. Izzo. 1993a. Mycobacterial virulence. Virulent strains of *Mycobacteria tuberculosis* have faster *in vivo* doubling times and are better equipped to resist growth-inhibiting functions of macrophages in the presence and absence of specific immunity. J.Exp.Med. **177**:1723-1733.

North, R. J. and A. A. Izzo. 1993b. Granuloma formation in severe combined immunodeficient (SCID) mice in response to progressive BCG infection. Tendency not to form granulomas in the lung is associated with faster bacterial growth in this organ. Am.J.Pathol. **142**:1959-1966.

O

O'Brien, R. J. and P. P. Nunn. 2001. The need for new drugs against tuberculosis. Obstacles, opportunities, and next steps. Am.J.Respir.Crit Care Med. **163**:1055-1058.

O'Donnell, M. A., A. Aldovini, R. B. Duda, H. Yang, A. Szilvasi, R. A. Young, and W. C. DeWolf. 1994. Recombinant *Mycobacterium bovis* BCG secreting functional interleukin-2 enhances gamma interferon production by splenocytes. Infect.Immun. **62**:2508-2514.

O'Farrell, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. J.Biol.Chem. **250**:4007-4021.

Odhiambo, J. A., M. W. Borgdorff, F. M. Kiambih, D. K. Kibuga, D. O. Kwamanga, L. Ng'ang'a, R. Agwanda, N. A. Kalisvaart, O. Misljenovic, N. J. Nagelkerke, and M. Bosman. 1999. Tuberculosis and the HIV epidemic: increasing annual risk of tuberculous infection in Kenya, 1986-1996. Am.J.Public Health **89**:1078-1082.

Okkels, L. M., I. Brock, F. Follmann, E. M. Agger, S. M. Arend, T. H. Ottenhoff, F. Oftung, I. Rosenkrands, and P. Andersen. 2003. PPE protein (Rv3873) from DNA segment RD1 of *Mycobacterium tuberculosis*: strong recognition of both specific T-cell epitopes and epitopes conserved within the PPE family. Infect.Immun. **71**:6116-6123.

Olsen, A. W., P. R. Hansen, A. Holm, and P. Andersen. 2000. Efficient protection against *Mycobacterium tuberculosis* by vaccination with a single subdominant epitope from the ESAT-6 antigen. Eur.J.Immunol. **30**:1724-1732.

Olsen, A. W., L. A. van Pinxteren, L. M. Okkels, P. B. Rasmussen, and P. Andersen. 2001. Protection of mice with a tuberculosis subunit vaccine based on a fusion protein of antigen 85b and esat-6. Infect.Immun. **69**:2773-2778.

Olsen, A. W. and P. Andersen. 2003. A novel TB vaccine; strategies to combat a complex pathogen. Immunol.Lett. **85**:207-211.

Ottenhoff, T. H., F. A. Verreck, E. G. Lichtenauer-Kaligis, M. A. Hoeve, O. Sanal, and J. T. van Dissel. 2002. Genetics, cytokines and human infectious disease: lessons from weakly pathogenic mycobacteria and salmonellae. Nat.Genet. **32**:97-105.

P

Pal, P. G. and M. A. Horwitz. 1992. Immunization with extracellular proteins of *Mycobacterium tuberculosis* induces cell-mediated immune responses and substantial protective immunity in a guinea pig model of pulmonary tuberculosis. Infect.Immun. **60**:4781-4792.

Park, H. D., K. M. Guinn, M. I. Harrell, R. Liao, M. I. Voskuil, M. Tompa, G. K. Schoolnik, and D. R. Sherman. 2003. Rv3133c/dosR is a transcription factor that mediates the hypoxic response of *Mycobacterium tuberculosis*. Mol.Microbiol. **48**:833-843.

Parrish, N. M., J. D. Dick, and W. R. Bishai. 1998. Mechanisms of latency in *Mycobacterium tuberculosis*. Trends Microbiol. **6**:107-112.

Pethe, K., S. Alonso, F. Biet, G. Delogu, M. J. Brennan, C. Locht, and F. D. Menozzi. 2001. The heparin-binding haemagglutinin of *M. tuberculosis* is required for extrapulmonary dissemination. Nature **412**:190-194.

Pfyffer, G. E., C. Cieslak, H. M. Welscher, P. Kissling, and S. Rusch-Gerdes. 1997a. Rapid detection of mycobacteria in clinical specimens by using the automated BACTEC 9000 MB system and comparison with radiometric and solid-culture systems. J.Clin.Microbiol. **35**:2229-2234.

Pfyffer, G. E., H. M. Welscher, P. Kissling, C. Cieslak, M. J. Casal, J. Gutierrez, and S. Rusch-Gerdes. 1997b. Comparison of the Mycobacteria Growth Indicator Tube (MGIT) with radiometric and solid culture for recovery of acid-fast bacilli. J.Clin.Microbiol. **35**:364-368.

Pheiffer, C., J. Betts, P. Lukey, and P. van Helden. 2002. Protein expression in *Mycobacterium tuberculosis* differs with growth stage and strain type. Clin.Chem.Lab Med. **40**:869-875.

Pollock, J. M. and P. Andersen. 1997. The potential of the ESAT-6 antigen secreted by virulent mycobacteria for specific diagnosis of tuberculosis. J.Infect.Dis. **175**:1251-1254.
Pottumarthy, S., V. C. Wells, and A. J. Morris. 2000. A comparison of seven tests for serological diagnosis of tuberculosis. J.Clin.Microbiol. **38**:2227-2231.
Power, C. A., G. Wei, and P. A. Bretscher. 1998. Mycobacterial dose defines the Th1/Th2 nature of the immune response independently of whether immunization is administered by the intravenous, subcutaneous, or intradermal route. Infect.Immun. **66**:5743-5750.
Punnonen, J. and J. E. de Vries. 1994. IL-13 induces proliferation, Ig isotype switching, and Ig synthesis by immature human fetal B cells. J.Immunol. **152**:1094-1102.
Pym, A. S., P. Brodin, L. Majlessi, R. Brosch, C. Demangel, A. Williams, K. E. Griffiths, G. Marchal, C. Leclerc, and S. T. Cole. 2003. Recombinant BCG exporting ESAT-6 confers enhanced protection against tuberculosis. Nat.Med. **9**:533-539.

R

Radhakrishnan, V. V. and A. Mathai. 1991. Detection of *Mycobacterium tuberculosis* antigen 5 in cerebrospinal fluid by inhibition ELISA and its diagnostic potential in tuberculous meningitis. J.Infect.Dis. **163**:650-652.
Raja, A., P. R. Narayanan, R. Mathew, and R. Prabhakar. 1995. Characterization of mycobacterial antigens and antibodies in circulating immune complexes from pulmonary tuberculosis. J.Lab Clin.Med. **125**:581-587.
Raja, A., K. R. Uma Devi, B. Ramalingam, and P. J. Brennan. 2002. Immunoglobulin G, A, and M responses in serum and circulating immune complexes elicited by the 16-kilodalton antigen of *Mycobacterium tuberculosis*. Clin.Diagn.Lab Immunol. **9**:308-312.
Ribeiro-Rodrigues, R., C. T. Resende, J. L. Johnson, F. Ribeiro, M. Palaci, R. T. Sa, E. L. Maciel, F. E. Pereira Lima, V. Dettoni, Z. Toossi, W. H. Boom, R. Dietze, J. J. Ellner, and C. S. Hirsch. 2002. Sputum cytokine levels in patients with pulmonary tuberculosis as early markers of mycobacterial clearance. Clin.Diagn.Lab Immunol. **9**:818-823.
Rieder, H. L. 1996. Sputum smear conversion during directly observed treatment for tuberculosis. Tuber.Lung Dis. **77**:124-129.
Riley, R. L., C. L. Mills, W. NYKA, N. Weinstock, P. B. Storey, L. K. Sultan, M. C. Riley, and W. F. Wells. 1959. Aerial dissemination of pulmonary tuberculosis: a two year study of contagion in a tuberculosis ward. Am.J.Hyg. **70**:185.
Rogers, P. R. and M. Croft. 2000. CD28, Ox-40, LFA-1, and CD4 modulation of Th1/Th2 differentiation is directly dependent on the dose of antigen. J.Immunol. **164**:2955-2963.
Rojas-Espinosa, O., J. Rangel-Moreno, A. Amador-Jimenez, R. Parra-Maldonado, P. Arce-Paredes, and J. Torres-Lopez. 1999. Secretion antigens of *Mycobacterium tuberculosis*: a comparison between a reference strain and seven wild isolates. Arch.Med.Res. **30**:171-178.
Rosenkrands, I., K. Weldingh, S. Jacobsen, C. V. Hansen, W. Florio, I. Gianetri, and P. Andersen. 2000a. Mapping and identification of *Mycobacterium tuberculosis* proteins by two-dimensional gel electrophoresis, microsequencing and immunodetection. Electrophoresis **21**:935-948.
Rosenkrands, I., A. King, K. Weldingh, M. Moniatte, E. Moertz, and P. Andersen. 2000b. Towards the proteome of *Mycobacterium tuberculosis*. Electrophoresis **21**:3740-3756.
Rosenkrands, I., R. A. Slayden, J. Crawford, C. Aagaard, C. E. Barry, III, and P. Andersen. 2002. Hypoxic response of *Mycobacterium tuberculosis* studied by metabolic labeling and proteome analysis of cellular and extracellular proteins. J.Bacteriol. **184**:3485-3491.
Rossouw, M., H. J. Nel, G. S. Cooke, P. D. van Helden, and E. G. Hoal. 2003. Association between tuberculosis and a polymorphic NFkappaB binding site in the interferon gamma gene. Lancet **361**:1871-1872.

S

Sada, E., G. M. Ruiz-Palacios, Y. Lopez-Vidal, and d. L. Ponce. 1983. Detection of mycobacterial antigens in cerebrospinal fluid of patients with tuberculous meningitis by enzyme-linked immunosorbent assay. Lancet **2**:651-652.

Sager, P., M. Schalimtzer, and V. Moller-Christensen. 1972. A case of spondylitis tuberculosa in the Danish Neolithic Age. Dan.Med.Bull. **19**:176-180.

Samanich, K., J. T. Belisle, and S. Laal. 2001. Homogeneity of antibody responses in tuberculosis patients. Infect.Immun. **69**:4600-4609.

Samanich, K. M., J. T. Belisle, M. G. Sonnenberg, M. A. Keen, S. Zolla-Pazner, and S. Laal. 1998. Delineation of human antibody responses to culture filtrate antigens of *Mycobacterium tuberculosis*. J.Infect.Dis. **178**:1534-1538.

Samanich, K. M., M. A. Keen, V. D. Vissa, J. D. Harder, J. S. Spencer, J. T. Belisle, S. Zolla-Pazner, and S. Laal. 2000. Serodiagnostic potential of culture filtrate antigens of *Mycobacterium tuberculosis*. Clin.Diagn.Lab Immunol. **7**:662-668.

Sampson, S. L., R. M. Warren, M. Richardson, G. D. Van Der Spuy, and P. D. van Helden. 1999. Disruption of coding regions by IS*6110* insertion in *Mycobacterium tuberculosis*. Tuber.Lung Dis. **79**:349-359.

Sampson, S. L., P. Lukey, R. M. Warren, P. D. van Helden, M. Richardson, and M. J. Everett. 2001. Expression, characterization and subcellular localization of the *Mycobacterium tuberculosis* PPE gene Rv1917c. Tuberculosis.(Edinb.) **81**:305-317.

Sánchez-Rodriguez, C., C. Estrada-Chavez, J. Garcia-Vigil, F. Laredo-Sanchez, J. Halabe-Cherem, A. Pereira-Suarez, and R. Mancilla. 2002. An IgG antibody response to the antigen 85 complex is associated with good outcome in Mexican Totonaca Indians with pulmonary tuberculosis. Int.J.Tuberc.Lung Dis. **6**:706-712.

Sander, B., U. Skansen-Saphir, O. Damm, L. Hakansson, J. Andersson, and U. Andersson. 1995. Sequential production of Th1 and Th2 cytokines in response to live bacillus Calmette-Guérin. Immunology **86**:512-518.

Saunders, B. M., A. A. Frank, and I. M. Orme. 1999. Granuloma formation is required to contain bacillus growth and delay mortality in mice chronically infected with *Mycobacterium tuberculosis*. Immunology **98**:324-328.

Saunders, B. M. and A. M. Cooper. 2000. Restraining mycobacteria: role of granulomas in mycobacterial infections. Immunol.Cell Biol. **78**:334-341.

Schlesinger, L. S., T. M. Kaufman, S. Iyer, S. R. Hull, and L. K. Marchiando. 1996. Differences in mannose receptor-mediated uptake of lipoarabinomannan from virulent and attenuated strains of *Mycobacterium tuberculosis* by human macrophages. J.Immunol. **157**:4568-4575.

Seder, R. A. and A. V. Hill. 2000. Vaccines against intracellular infections requiring cellular immunity. Nature **406**:793-798.

Seibert, F. B. and J. T. Glenn. 1941. Tuberculin purified protein derivative. Preparation and analysis of a large quantity for standard. Am.Rev.Tuberc. **44**:9-25.

Sethna, K. B., N. F. Mistry, Y. Dholakia, N. H. Antia, and M. Harboe. 1998. Longitudinal trends in serum levels of mycobacterial secretory (30 kD) and cytoplasmic (65 kD) antigens during chemotherapy of pulmonary tuberculosis patients. Scand.J.Infect.Dis. **30**:363-369.

Shafer, R. W., P. M. Small, C. Larkin, S. P. Singh, P. Kelly, M. F. Sierra, G. Schoolnik, and K. D. Chirgwin. 1995. Temporal trends and transmission patterns during the emergence of multidrug-resistant tuberculosis in New York City: a molecular epidemiologic assessment. J.Infect.Dis. **171**:170-176.

Shakib, F. and D. R. Stanworth. 1980. Human IgG subclasses in health and disease. (A review). Part I. Ric.Clin.Lab **10**:463-479.

Sherman, D. R., M. Voskuil, D. Schnappinger, R. Liao, M. I. Harrell, and G. K. Schoolnik. 2001. Regulation of the *Mycobacterium tuberculosis* hypoxic response gene encoding alpha -crystallin. Proc.Natl.Acad.Sci.U.S.A **98**:7534-7539.

Shinnick, T. M., M. H. Vodkin, and J. C. Williams. 1988. The *Mycobacterium tuberculosis* 65-kilodalton antigen is a heat shock protein which corresponds to common antigen and to the *Escherichia coli* GroEL protein. Infect.Immun. **56**:446-451.

Shinnick, T. M. and R. C. Good. 1994. Mycobacterial taxonomy. Eur.J.Clin.Microbiol.Infect.Dis. **13**:884-901.

Silva, C. L. 1999. The potential use of heat-shock proteins to vaccinate against mycobacterial infections. Microbes.Infect. **1**:429-435.

Silver, R. F., Q. Li, and J. J. Ellner. 1998. Expression of virulence of *Mycobacterium tuberculosis* within human monocytes: virulence correlates with intracellular growth and induction of tumor necrosis factor alpha but not with evasion of lymphocyte-dependent monocyte effector functions. Infect.Immun. **66**:1190-1199.

Skeiky, Y. A., P. J. Ovendale, S. Jen, M. R. Alderson, D. C. Dillon, S. Smith, C. B. Wilson, I. M. Orme, S. G. Reed, and A. Campos-Neto. 2000. T cell expression cloning of a *Mycobacterium tuberculosis* gene encoding a protective antigen associated with the early control of infection. J.Immunol. **165**:7140-7149.

Slobbe, L., E. Lockhart, M. A. O'Donnell, C. MacKintosh, G. De Lisle, and G. Buchan. 1999. An *in vivo* comparison of bacillus Calmette-Guérin (BCG) and cytokine-secreting BCG vaccines. Immunology **96**:517-523.

Smith, A. L., P. Hesketh, A. Archer, and M. W. Shirley. 2002. Antigenic diversity in *Eimeria maxima* and the influence of host genetics and immunization schedule on cross-protective immunity. Infect.Immun. **70**:2472-2479.

Smith, M. B., J. S. Bergmann, M. Onoroto, G. Mathews, and G. L. Woods. 1999. Evaluation of the enhanced amplified *Mycobacterium tuberculosis* direct test for direct detection of *Mycobacterium tuberculosis* complex in respiratory specimens. Arch.Pathol.Lab Med. **123**:1101-1103.

Smith, S. M. and H. M. Dockrell. 2000. Role of CD8 T cells in mycobacterial infections. Immunol.Cell Biol. **78**:325-333.

Snider, D. E., Jr. 1982. The tuberculin skin test. Am.Rev.Respir.Dis. **125**:108-118.

Sonnenberg, M. G. and J. T. Belisle. 1997. Definition of *Mycobacterium tuberculosis* culture filtrate proteins by two-dimensional polyacrylamide gel electrophoresis, N-terminal amino acid sequencing, and electrospray mass spectrometry. Infect.Immun. **65**:4515-4524.

Sorensen, A. L., S. Nagai, G. Houen, P. Andersen, and A. B. Andersen. 1995. Purification and characterization of a low-molecular-mass T-cell antigen secreted by *Mycobacterium tuberculosis.* Infect.Immun. **63**:1710-1717.

Sousa, A. O., A. Wargnier, Y. Poinsignon, N. Simonney, F. Gerber, F. Lavergne, J. L. Herrmann, and P. H. Lagrange. 2000. Kinetics of circulating antibodies, immune complex and specific antibody-secreting cells in tuberculosis patients during 6 months of antimicrobial therapy. Tuber.Lung Dis. **80**:27-33.

Spellberg, B. and J. E. Edwards, Jr. 2001. Type 1/Type 2 immunity in infectious diseases. Clin.Infect.Dis. **32**:76-102.

Springer, B., L. Stockman, K. Teschner, G. D. Roberts, and E. C. Bottger. 1996. Two-laboratory collaborative study on identification of mycobacteria: molecular versus phenotypic methods. J.Clin.Microbiol. **34**:296-303.

Sreevatsan, S., X. Pan, K. E. Stockbauer, N. D. Connell, B. N. Kreiswirth, T. S. Whittam, and J. M. Musser. 1997. Restricted structural gene polymorphism in the *Mycobacterium tuberculosis* complex indicates evolutionarily recent global dissemination. Proc.Natl.Acad.Sci.U.S.A **94**:9869-9874.

Stauffer, F., R. Mutschlechner, P. Hasenberger, S. Stadlbauer, and H. Schinko. 1995. Detection of *Mycobacterium tuberculosis* complex in clinical specimens by a commercial polymerase chain reaction kit. Eur.J.Clin.Microbiol.Infect.Dis. **14**:1046-1051.

Sterne, J. A., L. C. Rodrigues, and I. N. Guedes. 1998. Does the efficacy of BCG decline with time since vaccination? Int.J.Tuberc.Lung Dis. **2**:200-207.

Stewart, G. R., V. A. Snewin, G. Walzl, T. Hussell, P. Tormay, P. O'Gaora, M. Goyal, J. Betts, I. N. Brown, and D. B. Young. 2001. Overexpression of heat-shock proteins reduces survival of *Mycobacterium tuberculosis* in the chronic phase of infection. Nat.Med. **7**:732-737.

Sultan, L., W. Nyka, C. Mills, F. O'Grady, W. Wells, and R. L. Riley. 1960. Tuberculosis disseminators. A study of the variability of aerial infectivity of tuberculous patients. Am.Rev.Respir.Dis. **82**:358-369.

Supply, P., E. Mazars, S. Lesjean, V. Vincent, B. Gicquel, and C. Locht. 2000. Variable human minisatellite-like regions in the *Mycobacterium tuberculosis* genome. Mol.Microbiol. **36**:762-771.

T

Tabira, Y., N. Ohara, N. Ohara, H. Kitaura, S. Matsumoto, M. Naito, and T. Yamada. 1998. The 16-kDa alpha-crystallin-like protein of *Mycobacterium bovis* BCG is produced under conditions of oxygen deficiency and is associated with ribosomes. Res.Microbiol. **149**:255-264.

Talbot, E. A., M. D. Perkins, S. F. Silva, and R. Frothingham. 1997. Disseminated bacille Calmette-Guérin disease after vaccination: case report and review. Clin.Infect.Dis. **24**:1139-1146.

Talaat, A. M., P. Hunter, and S. A. Johnston. 2000. Genome-directed primers for selective labeling of bacterial transcripts for DNA microarray analysis. Nat.Biotechnol. **18**:679-682.

Tanzania Tuberculin Survey Collaboration. 2001. Tuberculosis control in the era of the HIV epidemic: risk of tuberculosis infection in Tanzania, 1983-1998. Int.J.Tuberc.Lung Disease **5**:103-112.

Tascon, R. E., M. J. Colston, S. Ragno, E. Stavropoulos, D. Gregory, and D. B. Lowrie. 1996. Vaccination against tuberculosis by DNA injection. Nat.Med. **2**:888-892.

Teitelbaum, R., A. Glatman-Freedman, B. Chen, J. B. Robbins, E. Unanue, A. Casadevall, and B. R. Bloom. 1998. A mAb recognizing a surface antigen of *Mycobacterium tuberculosis* enhances host survival. Proc.Natl.Acad.Sci.U.S.A **95**:15688-15693.

Tevere, V. J., P. L. Hewitt, A. Dare, P. Hocknell, A. Keen, J. P. Spadoro, and K. K. Young. 1996. Detection of *Mycobacterium tuberculosis* by PCR amplification with pan-*Mycobacterium* primers and hybridization to an *M. tuberculosis*-specific probe. J.Clin.Microbiol. **34**:918-923.

Toossi, Z. and J. J. Ellner. 1996. Mechanisms of anergy in tuberculosis. Curr.Top.Microbiol.Immunol. **215**:221-238.

Toossi, Z., J. L. Johnson, R. A. Kanost, M. Wu, H. Luzze, P. Peters, A. Okwera, M. Joloba, P. Mugyenyi, R. D. Mugerwa, H. Aung, J. J. Ellner, and C. S. Hirsch. 2001. Increased replication of HIV-1 at sites of *Mycobacterium tuberculosis* infection: potential mechanisms of viral activation. J.Acquir.Immune.Defic.Syndr. **28**:1-8.

Torres, M., P. Mendez-Sampeiro, L. Jimenez-Zamudio, L. Teran, A. Camarena, R. Quezada, E. Ramos, and E. Sada. 1994. Comparison of the immune response against *Mycobacterium tuberculosis* antigens between a group of patients with active pulmonary tuberculosis and healthy household contacts. Clin.Exp.Immunol. **96**:75-78.

Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc.Natl.Acad.Sci.U.S.A **76**:4350-4354.

Triccas, J. A., T. Parish, W. J. Britton, and B. Gicquel. 1998. An inducible expression system permitting the efficient purification of a recombinant antigen from *Mycobacterium smegmatis*. FEMS Microbiol.Lett. **167**:151-156.

Turner, O. C., A. D. Roberts, A. A. Frank, S. W. Phalen, D. M. McMurray, J. Content, O. Denis, S. D'Souza, A. Tanghe, K. Huygen, and I. M. Orme. 2000. Lack of protection in mice and necrotizing bronchointerstitial pneumonia with bronchiolitis in guinea pigs immunized with vaccines directed against the hsp60 molecule of *Mycobacterium tuberculosis*. Infect.Immun. **68**:3674-3679.

U

Ulrichs, T., M. E. Munk, H. Mollenkopf, S. Behr-Perst, R. Colangeli, M. L. Gennaro, and S. H. Kaufmann. 1998. Differential T cell responses to *Mycobacterium tuberculosis* ESAT6 in tuberculosis patients and healthy donors. Eur.J.Immunol. **28**:3949-3958.

Ulrichs, T., P. Anding, S. Porcelli, S. H. Kaufmann, and M. E. Munk. 2000. Increased numbers of ESAT-6- and purified protein derivative-specific gamma interferon-producing cells in subclinical and active tuberculosis infection. Infect.Immun. **68**:6073-6076.

Urquhart, B. L., T. E. Atsalos, D. Roach, D. J. Basseal, B. Bjellqvist, W. L. Britton, and I. Humphery-Smith. 1997. 'Proteomic contigs' of *Mycobacterium tuberculosis* and *Mycobacterium bovis* (BCG) using novel immobilised pH gradients. Electrophoresis **18**:1384-1392.

V

Valway, S. E., M. P. Sanchez, T. F. Shinnick, I. Orme, T. Agerton, D. Hoy, J. S. Jones, H. Westmoreland, and I. M. Onorato. 1998. An outbreak involving extensive transmission of a virulent strain of *Mycobacterium tuberculosis*. N.Engl.J.Med. **338**:633-639.
van Embden, J. D., M. D. Cave, J. T. Crawford, J. W. Dale, K. D. Eisenach, B. Gicquel, P. Hermans, C. Martin, R. McAdam, and T. M. Shinnick. 1993. Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: recommendations for a standardized methodology. J.Clin.Microbiol. **31**:406-409.
van Helden, P. D., R. M. Warren, T. C. Victor, S. G. van der, M. Richardson, and E. Hoal-van Helden. 2002. Strain families of *Mycobacterium tuberculosis*. Trends Microbiol. **10**:167-168.
van Pinxteren, L. A., J. P. Cassidy, B. H. Smedegaard, E. M. Agger, and P. Andersen. 2000. Control of latent *Mycobacterium tuberculosis* infection is dependent on CD8 T cells. Eur.J.Immunol. **30**:3689-3698.
van Rie, A., R. Warren, M. Richardson, T. C. Victor, R. P. Gie, D. A. Enarson, N. Beyers, and P. D. van Helden. 1999a. Exogenous reinfection as a cause of recurrent tuberculosis after curative treatment. N.Engl.J.Med. **341**:1174-1179.
van Rie, A., R. M. Warren, N. Beyers, R. P. Gie, C. N. Classen, M. Richardson, S. L. Sampson, T. C. Victor, and P. D. van Helden. 1999b. Transmission of a multidrug-resistant *Mycobacterium tuberculosis* strain resembling "strain W" among noninstitutionalized, human immunodeficiency virus-seronegative patients. J.Infect.Dis. **180**:1608-1615.
van Soolingen, D., L. Qian, P. E. de Haas, J. T. Douglas, H. Traore, F. Portaels, H. Z. Qing, D. Enkhsaikan, P. Nymadawa, and J. D. van Embden. 1995. Predominance of a single genotype of *Mycobacterium tuberculosis* in countries of east Asia. J.Clin.Microbiol. **33**:3234-3238.
Verbon, A., R. A. Hartskeerl, C. Moreno, and A. H. Kolk. 1992. Characterization of B cell epitopes on the 16K antigen of *Mycobacterium tuberculosis*. Clin.Exp.Immunol. **89**:395-401.

W

Wallis, R. S., M. Perkins, M. Phillips, M. Joloba, B. Demchuk, A. Namale, J. L. Johnson, D. Williams, K. Wolski, L. Teixeira, R. Dietze, R. D. Mugerwa, K. Eisenach, and J. J. Ellner. 1998. Induction of the antigen 85 complex of *Mycobacterium tuberculosis* in sputum: a determinant of outcome in pulmonary tuberculosis treatment. J.Infect.Dis. **178**:1115-1121.
Wallis, R. S., S. Patil, S. H. Cheon, K. Edmonds, M. Phillips, M. D. Perkins, M. Joloba, A. Namale, J. L. Johnson, L. Teixeira, R. Dietze, S. Siddiqi, R. D. Mugerwa, K. Eisenach, and J. J. Ellner. 1999. Drug tolerance in *Mycobacterium tuberculosis*. Antimicrob.Agents Chemother. **43**:2600-2606.
Warren, R. M., S. L. Sampson, M. Richardson, G. D. Van Der Spuy, C. J. Lombard, T. C. Victor, and P. D. van Helden. 2000. Mapping of IS*6110* flanking regions in clinical isolates of *Mycobacterium tuberculosis* demonstrates genome plasticity. Mol.Microbiol. **37**:1405-1416.
Wasinger, V. C., S. J. Cordwell, A. Cerpa-Poljak, J. X. Yan, A. A. Gooley, M. R. Wilkins, M. W. Duncan, R. Harris, K. L. Williams, and I. Humphery-Smith. 1995. Progress with gene-product mapping of the Mollicutes: *Mycoplasma genitalium*. Electrophoresis **16**:1090-1094.
Wayne, L. G. 1994. Dormancy of *Mycobacterium tuberculosis* and latency of disease. Eur.J.Clin.Microbiol.Infect.Dis. **13**:908-914.
Wayne, L. G. and H. A. Sramek. 1994. Metronidazole is bactericidal to dormant cells of *Mycobacterium tuberculosis*. Antimicrob.Agents Chemother. **38**:2054-2058.
Wayne, L. G. and L. G. Hayes. 1996. An *in vitro* model for sequential study of shiftdown of *Mycobacterium tuberculosis* through two stages of nonreplicating persistence. Infect.Immun. **64**:2062-2069.
Weill-Hallé, B. and R. Turpin. 1925. Premiers essais de vaccination antituberculeuse de l'enfant par le bacille Calmette-Guérin (BCG). Bull Mem Soc Med l'Hosp de Paris **49**:1589-1601.
Weiser, J. N., J. M. Love, and E. R. Moxon. 1989. The molecular mechanism of phase variation of *H. influenzae* lipopolysaccharide. Cell **59**:657-665.
WHO. 1993. WHO declares tuberculosis a global emergency. Soz.Praventivmed **38**:251-252.
WHO. 2002. Global tuberculosis Control. WHO/CDS/TB/2002.295.
WHO. 2003. Global tuberculosis Control. WHO/CDS/TB/2003.316.

Wilkins, E. G. L. 1994. The serodiagnosis of tuberculosis, p. 365-378. *In* P. D. O. Davies (ed.), Clinical tuberculosis. Chapman and Hall, London.
Wilkins, E. G. and J. Ivanyi. 1990. Potential value of serology for diagnosis of extrapulmonary tuberculosis. Lancet **336**:641-644.
Wilkinson, R. J., K. Haslov, R. Rappuoli, F. Giovannoni, P. R. Narayanan, C. R. Desai, H. M. Vordermeier, J. Paulsen, G. Pasvol, J. Ivanyi, and M. Singh. 1997. Evaluation of the recombinant 38-kilodalton antigen of *Mycobacterium tuberculosis* as a potential immunodiagnostic reagent. J.Clin.Microbiol. **35**:553-557.
Wilkinson, R. J., K. A. Wilkinson, K. A. De Smet, K. Haslov, G. Pasvol, M. Singh, I. Svarcova, and J. Ivanyi. 1998. Human T- and B-cell reactivity to the 16kDa alpha-crystallin protein of *Mycobacterium tuberculosis*. Scand.J.Immunol. **48**:403-409.

Y

Yeremeev, V. V., I. V. Lyadova, B. V. Nikonenko, A. S. Apt, C. Abou-Zeid, J. Inwald, and D. B. Young. 2000. The 19-kD antigen and protective immunity in a murine model of tuberculosis. Clin.Exp.Immunol. **120**:274-279.
Young, S. L., M. A. O'Donnell, and G. S. Buchan. 2002. IL-2-secreting recombinant bacillus Calmette Guerin can overcome a Type 2 immune response and corticosteroid-induced immunosuppression to elicit a Type 1 immune response. Int.Immunol. **14**:793-800.
Yuan, Y., D. D. Crane, and C. E. Barry, III. 1996. Stationary phase-associated protein expression in *Mycobacterium tuberculosis*: function of the mycobacterial alpha-crystallin homolog. J.Bacteriol. **178**:4484-4492.
Yuan, Y., D. D. Crane, R. M. Simpson, Y. Q. Zhu, M. J. Hickey, D. R. Sherman, and C. E. Barry, III. 1998. The 16-kDa alpha-crystallin (Acr) protein of *Mycobacterium tuberculosis* is required for growth in macrophages. Proc.Natl.Acad.Sci.U.S.A **95**:9578-9583.

Z

Zahrt, T. C. and V. Deretic. 2002. Reactive nitrogen and oxygen intermediates and bacterial defenses: unusual adaptations in *Mycobacterium tuberculosis*. Antioxid.Redox.Signal. **4**:141-159.
Zhang, M., J. Gong, Z. Yang, B. Samten, M. D. Cave, and P. F. Barnes. 1999. Enhanced capacity of a widespread strain of *Mycobacterium tuberculosis* to grow in human macrophages. J.Infect.Dis. **179** :1213-1217.
Zhang, Q. and K. S. Wise. 2001. Coupled phase-variable expression and epitope masking of selective surface lipoproteins increase surface phenotypic diversity in *Mycoplasma hominis*. Infect.Immun. **69**:5177-5181.